Effect of Murine Kidney Extracts on the Proliferation of Hematopoietic Progenitor Cells in Human Umbilical Cord Blood

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We examined the effect of murine kidney extract (MKE) on the clonal growth of highly purified CD34$^+$ hematopoietic progenitor cells from human umbilical cord blood. MKE did not affect the total number of colonies of erythroid burst-forming units (BFU-E), granulocyte-macrophage colony-forming units (CFU-GM) or granulocyte-erythroid-macrophage-megakaryocyte colony-forming units (CFU-Mix/CFU-GEMM) in a methylcellulose culture with exogenous recombinant human granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin-3, stem cell factor and erythropoietin. MKE significantly increased the proportion of BFU-E- or CFU-Mix-derived colonies, and suppressed the formation CFU-GM-derived colonies depending on the MKE dose. However, because of an increase in small megakaryocyte colonies derived from mature CFU-Meg MKE increased by approximately 40% the growth of megakaryocyte colony-forming units (CFU-Meg) in plasma clot culture stimulated by recombinant human thrombopoietin. Also MKE promoted an increase in hyperploid megakaryocytes, suggesting that the active factor(s) in MKE acts on the mature CFU-Meg and promotes the maturation of megakaryocytes. Gel-filtration high performance liquid chromatography of MKE showed that the promoting factor(s) in MKE was approximately 45 kDa. These results indicate that the factor(s) detected in MKE influence human hematopoiesis *in vitro*, especially thrombopoiesis.

Key words kidney; megakaryocyte; CD34$^+$ cells; umbilical cord blood

Blood cells, such as neutrophils, erythrocytes and lymphocytes, are produced by the proliferation and differentiation of hematopoietic stem cells. Hematopoiesis is regulated by a complex system of positive and negative regulators, some of which are produced by various kinds of cells from bone marrow-, fibroblasts, endothelial cells, stromal cells, macrophages and lymphocytes, *etc.*[1,2] The kidney comprises numerous cells that have various functions, and is one of the most important tissues in hematopoiesis. The kidney is involved in the mechanism of oxygen sensing. It produces erythropoietin (Epo), which usually acts on relatively mature erythroid precursor cells, differentiating them into erythrocytes[3] in proximal renal tubular cells in normal mice.[4,5]

We reported previously that aqueous normal murine kidney extracts (MKE) show colony-promoting activity (CPA) of murine hematopoietic progenitor cells in the presence of interleukin-3 (IL-3) plus Epo *in vitro*.[6] MKE preferentially enhances granulocyte-macrophage colony-forming units (CFU-GM), but does not promote erythroid colony formation and has no colony-stimulating activity by itself. We reported also that MKE contains regulators that suppress the growth of murine mast cells and histamine synthesis.[7] This study suggests the possibility that mast cell inhibitory factor(s) in MKE are different from colony-promoting factor(s), and that MKE may contain different regulators for hematopoiesis, proliferation and differentiation of CFU-GM and mast cells. To understand the activity of MKE in human hematopoiesis, we examined the effect of MKE on the clonal growth of highly purified CD34$^+$ hematopoietic progenitor cells from human umbilical cord blood (CB) in this study.

MATERIALS AND METHODS

Preparation of MKE MKE was prepared as described by Bamba *et al.*[6] Briefly, kidneys were removed from mice and were homogenized with distilled water at a concentration of 12% (w/v) using a Polytron (Kinematica, Switzerland) at 6×10^3 rpm for 30 s at three 30 s intervals at 0 °C. The homogenates were stirred for one night at 4 °C and were centrifuged at 9×10^3 ***g*** at 4 °C for 30 min. The supernatants were adjusted to pH 4.0 with acetic acid and insoluble material was removed by centrifugation. The clear supernatant was dialyzed against Dulbecco's phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}), was concentrated to 1/5 volume using an ultrafiltration membrane (molecular weight cut off: 10000, Amicon) and was stored at −20 °C until used.

Cytokines and Antibodies Recombinant human thrombopoietin (TPO), human IL-3, and human stem cell factor (SCF) were kindly provided by Kirin Brewery Co., Ltd. (Tokyo). Recombinant human granulocyte colony-stimulating factor (G-CSF) and Epo were purchased from Sankyo Co., Ltd. (Tokyo). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was kindly provided by Schering-Plough Co., Ltd. (Ireland). Doses of these factors per milliliter were: TPO, 50 ng; SCF, 100 ng; IL-3, 5 ng; G-CSF, 10 ng; GM-CSF, 10 ng; and Epo, 4 U. Fluorescence-labeled monoclonal antibody (MoAb), fluorescein isothiocyanate (FITC)-conjugated antihuman CD34 (FITC-CD34), FITC-conjugated antihuman CD41 (FITC-CD41), phycoerythrin (PE)-conjugated antihuman CD41 (PE-CD41), PE-cyanin 5 fluorochrome tandem (PC5)-conjugated antihuman CD45 (PC5-CD45) were purchased from Beckman Coulter Immunotech (Marseille, France)

Collection of CB and Isolation of CD34$^+$ Cells CB was collected at the end of full-term deliveries, after obtaining informed consent from the mothers, using a sterile collection bag containing anticoagulant citrate-phosphate dextrose according to the guidelines of the Tokyo Cord Blood

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Bank. Low-density mononuclear CB cells were separated by centrifugation using a Lymphosepar I (1.077 g/ml, IBL, Gunma, Japan) for 30 min at 300 ***g*** at room temperature, and were then washed twice with PBS containing 5 mM EDTA. Low-density mononuclear cells were processed for $CD34^+$ cell enrichment using a magnetic cell sorting MACS $CD34^+$ progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) that made a positive selection of $CD34^+$ cells. At the end of the procedure, $CD34^+$ cell recovery was 0.13—0.66%, and the purity was in the range of 88—93%, as measured using a flow cytometer (EPICS XL, Beckman Coulter, Fullerton, CA, U.S.A.).

Methylcellulose Cultures Colony-forming cells, including erythroid burst-forming units (BFU-E), CFU-GM and granulocyte-erythroid-macrophage-megakaryocyte colony-forming units (CFU-Mix/CFU-GEMM) were assayed using methylcellulose culture as described by Kashiwakura *et al.*[8] and Takahashi *et al.*[9] with minor modifications. $CD34^+$ cells at a concentration of 3×10^2 were plated in a 35 mm plastic plate (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in 1 ml Iscove's modified Dulbecco's medium (IMDM, GIBCO BRL, Life Technologies, Inc., Rockville, MD, U.S.A.) supplemented with 0.8% methylcellulose (Dow Chemical Co., Midland, MI, U.S.A.), 1% deionized bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) , 30% fetal calf serum (Intergen Co., Purchase, NY, U.S.A.), G-CSF, GM-CSF, IL-3, SCF and Epo. Each plate was incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 14 d. Colonies consisting of more than 50 cells were counted using an inverted microscope.

Plasma Clot Cultures of Megakaryocyte Progenitor Cells Megakaryocyte colony-forming units (CFU-Meg) were assayed using the plasma clot technique and platelet-poor human plasma as described by Kashiwakura *et al.*[10] The cultures contained a 1×10^3/ml concentration of $CD34^+$ cells, as well as 15% human AB blood-type platelet-poor plasma and TPO in IMDM with additives of 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1% MEM vitamin, 1% MEM nonessential amino acids (all from GIBCO BRL), 1×10^{-5}M thioglycerol (Sigma), 2 mg/ml L-asparagine (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), 1 mg/ml $CaCl_2$ (Wako), and 0.2% BSA. The medium (0.3 ml) was plated in wells of 24-well plates (Falcon) and was incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 10 d.

Immunofluorescence Staining to Identify Megakaryocyte Colonies Each well was fixed twice with a 2 : 1 mixture of acetone and methanol for 15 min. The plates were dried in air overnight and were then kept at −20 °C until staining. For the staining, the plates were taken out of the freezer and were returned to room temperature. Then, PBS containing 0.5% BSA (PBS-B) was added to soften the clot. The solution was discarded, FITC-CD41 MoAb diluted 1 : 100 in PBS-B was added, and the plates were incubated for 1 h at room temperature. The plates were washed and the nuclei were counterstained with 0.3 ng/ml propidium iodide (PI, Sigma). The colonies were washed again and were counted using a fluorescence microscope (Olympus, Tokyo) at a magnification of 100×. Megakaryocyte colonies were classified into 2 types: large colonies of more than 50 cells (immature CFU-Meg) and small colonies of 3 to 50 cells (mature CFU-Meg).[11,12] The total number of CFU-Meg (total CFU-Meg) was obtained by counting both immature CFU-Meg and mature CFU-Meg.

Determination of Megakaryocyte DNA Ploidy Megakaryocyte DNA ploidy was determined using the method of Hagiwara *et al.*[13] with modifications. Cells before cultivation or cultured cells harvested from plasma clot culture were centrifuged for 5 min at 250 ***g***, and the cell pellets were resuspended in PBS-B containing 5 mM EDTA and were incubated with FITC-CD41 MoAb for 20 min at room temperature. Then the cells were washed with PBS and the cell pellets were resuspended in modified CATCH medium[14] containing 3.5% BSA and 0.5% Tween 20 (Wako). Following a 1 h incubation at 4 °C, the cells were fixed for 5 min by adding an equal volume of the same medium supplemented with 1% paraformaldehyde (Wako) to the cell suspension. After washing with PBS, the cells were resuspended in 50 μg/ml of PI dissolved in 0.7% citric acid and 0.6% NaCl, and were incubated for 1 h at 4 °C. After incubation, the cells were further incubated with 50 μg/ml of RNase (Sigma) for 30 min at room temperature. The cells were then passed through a 35 μm nylon mesh and were analyzed using a flow cytometer. The ploidy distribution was determined by setting markers at the nadirs between peaks using the 2N and 4N peaks of freshly isolated human CB mononuclear cells as standards.

Immunological Marker Analysis The expression of cell surface antigens on cells was analyzed using direct immunofluorescence flow cytometry using triple-staining combinations of MoAbs including PC5-CD45, FITC-CD34 and PE-CD41. Briefly, the cells were incubated with saturated concentrations of the relevant MoAbs for 20 min at room temperature, were washed, and were analyzed using a flow cytometer. For each experiment, negative controls were performed using isotype-matched irrelevant control MoAbs.

Trypsin and Heat Treatments Treatments with trypsin (10 mg/ml) were performed using the method of Brennan *et al.*[15] MKE was heated at 56 °C for 30 min, 70 °C for 30 min, and 100 °C for 2 min, and then insoluble materials were removed by centrifugation. Each treated MKE sample was assayed for activity at a value corresponding to 304 μg/ml of non-treated MKE.

Gel-Filtration HPLC (GF-HPLC) MKE was applied to a TSKgel $G3000SW_{XL}$ column (7.5 mm i.d.×30 cm, Tosoh) previously equilibrated with PBS at a flow rate of 30 ml/h. Fractions of 0.5 ml were collected, and the absorbance at 280 nm and the activity of each fraction were measured.

Protein Assay The protein contents of the samples were measured using Lowry's method [16] with BSA as the standard protein. MKE contained 15.2 mg protein /ml. Doses of MKE were represented by the concentration of protein as BSA.

Statistical Analysis The results were expressed as the mean for data from 2 or more separate experiments. The significance of difference between groups was determined using the Student's *t*-test.

RESULTS

Effect of MKE on the Growth of CB $CD34^+$ BFU-E, CFU-GM and CFU-Mix CB $CD34^+$ cells were assayed for BFU-E, CFU-GM and CFU-Mix using methylcellulose

cultures supplemented with recombinant human G-CSF, GM-CSF, IL-3, SCF and Epo. The average number of total colonies from 3 separate triplicate cultures was observed at 82.3±18.2 colonies per 300 CB $CD34^+$ cells, including 47.4 colonies of CFU-GM, 10.7 colonies of BFU-E and 24.3 colonies of CFU-Mix (Table 1). No significant increase in total colonies was observed by adding MKE (152—608 μg) (Fig. 1). However, MKE significantly increased the proportion of BFU-E- or CFU-Mix-derived colonies, and suppressed the formation CFU-GM-derived colonies depending on the MKE dose (Table 1). MKE alone supported no colony formation in the culture without exogenous cytokines.

Effect of MKE on the Growth of CB $CD34^+$ CFU-Meg We investigated the effect of MKE with or without TPO in plasma clot culture (Fig. 2). TPO alone supported approximately 83 megakaryocyte colonies per 1×10^3 CB $CD34^+$ cells in the plasma clot culture (Table 2), and adding MKE (304 μg) resulted in a significant increase (1.4-fold) in the total number of colonies observed. With both TPO alone or adding MKE, no colonies were observed except megakaryocyte colonies. In the culture with TPO alone, the proportion of large megakaryocyte colonies, which were immature CFU-Meg, was approximately 60% of the total number of colonies. MKE significantly promoted the formation of small megakaryocyte colonies, which were mature CFU-Meg and comprised 48—76% of the total number of colonies in the culture with MKE. In the culture without exogenous TPO, no colonies were observed in the presence or absence of MKE.

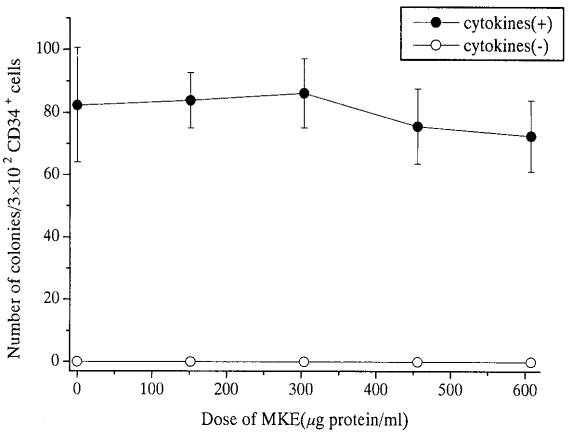

Fig. 1. Effect of MKE on the Growth of CB $CD34^+$ CFU-GM, BFU-E and CFU-Mix in Methylcellulose Culture

CFU-GM-,BFU-E and CFU-Mix-derived colonies were assayed with 3×10^2 CB $CD34^+$ cells with recombinant human G-CSF (10 ng/ml), GM-CSF (10 ng/ml), IL-3 (5 ng/ml), SCF (100 ng/ml) and Epo (4 U/ml). The culture was performed at 37 °C for 14 d. Values represent the mean±S.D. of 3 separate experiments with triplicate culture.

Flow Cytometric Analysis of Cell Surface Antigens and DNA Ploidy Analysis in the Cells Generated in the Plasma Clot Culture To evaluate the action of MKE on the proliferation and differentiation of CB $CD34^+$ cells, the surface antigen and DNA ploidy in the cells harvested from the culture were analyzed using a flow cytometer. Using anti-$CD45^+$ fluorescence antibody to identify normal human cells, the expression of CD34 or CD41 or both was assessed. Positive expressions of CD45 and CD34, CD45 and CD41 and CD34 and CD41 in the cells before culture were 87.16%, 0.48% and 1.68%, respectively (Fig. 3, Table 3). Each value of these cells in control cultures was 11.90%, 72.74% and 0.02%, respectively, suggesting that the hematopoietic stem cells rapidly decreased, while megakaryocytes increased in this culture condition. Similarly, almost the same values were found in the culture with MKE, but MKE supported $CD45^+$ and $CD34^+$ cells and $CD34^+$ and $CD41^+$ cells slightly higher than the control.

The DNA ploidy of $CD41^+$ cells was analyzed. 70.3% of the megakaryocytes were 2N ploidy and 5.0% of hyperploid megakaryocytes at more than 8 N ploidy were among the total megakaryocytes detected in the control culture (Fig. 4, Table 4). By adding MKE, megakaryocytes with 2N ploidy decreased to 55% and hyperploidy megakaryocytes increased to 9.4% of the total megakaryocytes. These results indicate that MKE promotes the maturation of megakaryocytes.

Trypsin and Heat Treatments The active molecule(s) was sensitive to trypsin, and was resistant to heat treatment at 70 °C for 30 min (Table 5). This molecule(s) seems to be a heat-stable protein.

Partial Purification of Megakaryocyte Colony-Promoting Factor(s) from MKE The molecular weight of active factor(s) in MKE was measured using GF-HPLC with a $G3000SW_{XL}$ column. The CPA of CFU-Meg was detected in a fraction eluted for 19—21 min (Fig. 5). The CPA of murine CFU-GM was also detected in the same fraction (data not shown). The molecular weight of this activity was about 45 kDa from the results of calibration using standard proteins (data not shown).

Table 1. Effect of MKE on Colony Proportion of CB $CD34^+$ CFU-GM, BFU-E and CFU-Mix

MKE (μg protein/ml)	Number of colonies/3×10^2 CB $CD34^+$ cells				Ratio
	CFU-GM	BFU-E	CFU-Mix	Total	
0	47.4±10.1 (57.6%)	10.7±4.6 (13.0%)	24.3±6.3 (29.4%)	82.3±18.2 (100%)	1.00
152	41.6±6.8 (49.6%)	12.1±4.1 (14.4%)	30.2±4.9* (36.0%)	83.9±8.8 (100%)	1.02
304	38.2±9.3 (44.3%)	17.7±4.5** (20.5%)	30.3±5.2* (35.2%)	86.2±11.0 (100%)	1.05
456	33.7±7.6*** (44.6%)	14.7±3.5 (19.4%)	27.2±4.3 (36.0%)	75.6±12.0 (100%)	0.92
608	31.4±11.2** (43.3%)	14.3±3.4 (19.7%)	26.9±6.9 (37.0%)	75.6±11.4 (100%)	0.88

Values in parentheses mean the proportion of colonies per total number of colonies. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

DISCUSSION

We investigated the *in vitro* action of MKE on the clonal growth of hematopoietic progenitor cells from human CB. The MKE increased the proportion of BFU-E or CFU-Mix or both and suppressed the growth of CFU-GM depending on the MKE dose, but no increase was observed in the total number of colonies in methylcellulose culture with exogenous recombinant human G-CSF, GM-CSF, IL-3, SCF, and Epo. However, we found that MKE significantly promoted the colony formation of CFU-Meg in plasma clot culture with recombinant human TPO alone.

In this study, we used highly purified $CD34^+$ cells from CB and human plasma to estimate the MKE action. The CB $CD34^+$ hematopoietic progenitor population is not homogenous, but includes lineage-committed progenitors, early progenitors[17,18] and stromal cells.[19] Some of these cells might produce cytokines. Also, human plasma may contain physiological regulators or growth factors that influence CFU-Meg growth in the culture.[20—22] However, no colonies were observed in the methylcellulose culture or the plasma clot culture without adding exogenous cytokines in spite of the presence or absence of MKE (Figs. 1, 2). Thus, estimating the MKE action on the growth of $CD34^+$ hematopoietic progenitor cells was negligibly influenced by the heterogenous property of $CD34^+$ cells and contaminants in human plasma in this study.

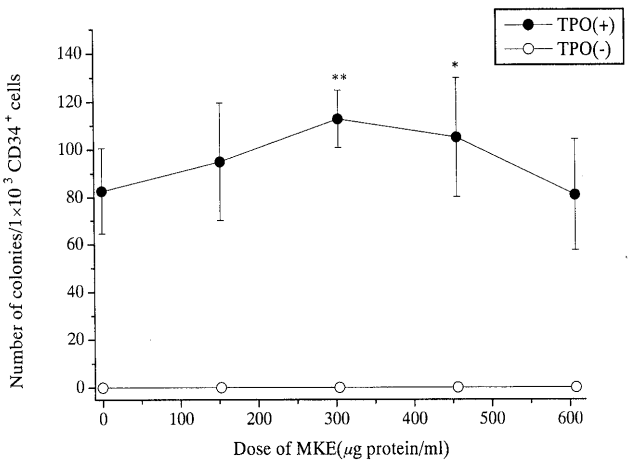

Fig. 2. Effect of MKE on the Growth of CB $CD34^+$ CFU-Meg in Plasma Clot Culture

Megakaryocyte colonies were assayed with 1×10^3 CB $CD34^+$ cells with recombinant human TPO. Values represent the mean±S.D. of 3 separate experiments with triplicate culture. *, $p<0.05$; **, $p<0.005$.

Thrombopoiesis *in vitro* requires at least a growth stimulus, termed megakaryocyte colony-stimulating factor (Meg-CSF), and other factors to develop megakaryocyte maturation.[23,24] Meg-CSF is required to proliferate CFU-Meg, colony induction and cell division, while a second factor, megakaryocyte potentiate factor (Meg-POT), influences DNA ploidy and acetylcholinesterase,[25] as well as the cell size of the developing megakaryocytes. IL-3 is a potent stimulant of CFU-Meg proliferation, but has little effect on megakaryocyte maturation.[26] SCF, IL-6, IL-11 and leukemia inhibitory factor do not act as Meg-CSFs as single agents *in vitro*, but can increase the effect of IL-3 on megakaryocyte colony formation, acting predominantly on megakaryocyte maturation.[27—31] Epo slightly increases both CFU-Meg proliferation and megakaryocyte maturation.[32,33] A ligand for the cytokine receptor protein (Mpl) encoded by *c-mpl* promotes the proliferation of CFU-Meg *in vitro* and *in vivo*.[34—36] This ligand protein has been termed Mpl ligand,[34] TPO,[35] or megakaryocyte growth and development factor.[36] TPO also increases the number of BFU-E, CFU-GM and CFU-Mix, as well as CFU-Meg *in vitro* and *in vivo*, and acts synergistically with other cytokines, including IL-3, IL-11, flt3-ligand (FL), G-CSF and SCF.[37—39] As MKE alone did not support megakaryocyte colonies formation in this study, the active factor(s) in MKE for human thrombopoiesis may be classified as Meg-POT. MKE selectively promoted small megakaryocyte colonies (Table 2) and increased hyperploid megakaryocytes of more than 8N (Table 3), suggesting that MKE acts on mature CFU-Meg and promotes the maturation of megakaryocytes.

Hematopoietic stem/progenitor cells in human CB are more immature and exist in higher concentrations than those in human bone marrow.[40—43] However, although CB has a higher clonogenic potential and is a better source of CFU-Meg than bone marrow or peripheral blood,[44] a long period of severe thrombocytopenia seems to be a problem in many cases after CB transplantation.[45] Transplantation with committed CFU-Meg may shorten the post-transplant thrombocytopenic period, as has been shown by peripheral blood stem cell transplantation.[46,47] The combination of TPO with SCF and FL results in amplifying CFU-Meg from CB in a liquid culture system.[48,49] Most cultured megakaryocytes, however, are populations with a lower ploidy, 2N and 4N

Table 2. Effect of MKE on the Growth of Immature and Mature CFU-Meg

MKE (μg protein/ml)	Number of colonies/1×10^3 CB $CD34^+$ cells			Ratio
	Immature CFU-Meg	Mature CFU-Meg	Total	
0	48.1±11.7 (58.3%)	34.4±8.2 (41.7%)	82.6±18.0 (100%)	1.00
152	49.3±14.0 (52.0%)	45.6±15.0 (48.0%)	94.8±24.7 (100%)	1.15
304	44.1±11.3 (39.1%)	68.5±10.8*** (60.9%)	112.6±12.0** (100%)	1.36**
456	30.0±10.8** (28.6%)	74.8±17.9*** (71.4%)	104.8±24.8* (100%)	1.27*
608	19.6±8.7*** (24.3%)	61.1±16.7** (75.7%)	80.7±23.3 (100%)	0.98

Values in parentheses mean the proportion of colonies per total colonies. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

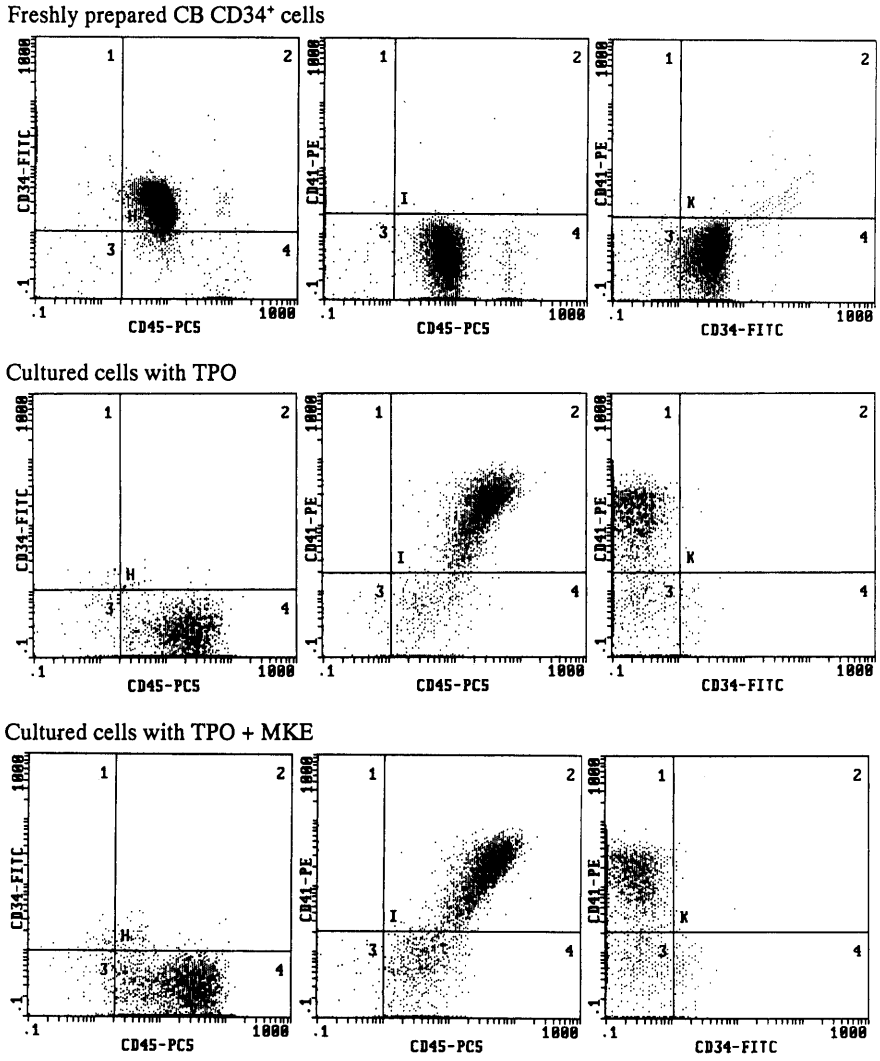

Fig. 3. Representative Flow Cytometric Analysis of Purified CB $CD34^+$ Cells and after 10 d of Stimulation with TPO Alone or TPO+MKE

Purified CB $CD34^+$ cells (1×10^3/ml) were cultured in a plasma clot with or without MKE (304 μg protein/ml) for 10 d, then the cells were harvested from the culture and were treated with anti-human FITC-CD34, PE-CD41 and PC5-CD45 MoAb. The expression of the surface antigen was analyzed using a flow cytometer.

Table 3. Expression of Surface Antigens in the Cells Generated in the Plasma Clot

Surface antigens	% of positive cells		
	day 0	day 10	
		Control	MKE
$CD45^+$, $CD34^+$	87.16±5.33	11.9±10.03	19.05±8.88
$CD45^+$, $CD41^+$	0.48±0.26	72.74±3.34	67.79±6.16
$CD34^+$, $CD41^+$	1.68±0.72	0.02±0.03	0.08±0.11

Purified CB $CD34^+$ cells (1×10^3/ml) were cultured in a plasma clot with or without MKE (304 μg protein/ml) for 10 d, then the cells were harvested from the culture and were treated with anti-human FITC-CD34, PE-CD41 and PC5-CD45 MoAb. The expression of the surface antigen was analyzed using a flow cytometer. Values are the mean±S.D. of 3 separate experiments.

Table 4. DNA Ploidy Distributions of $CD41^+$ Cells Generated with TPO Alone or TPO+MKE

Samples	DNA ploidy			
	2N	4N	8N	16N
Freshly prepared CB $CD34^+$cells	95.4%	3.9%	0.7%	0%
Cultured cells with TPO	70.3%	24.7%	4.5%	0.5%
Cultured cells with TPO +MKE (304 μg)	55.0%	35.6%	7.5%	1.9%

Purified CB $CD34^+$ cells (1×10^3/ml) were cultured in a plasma clot with or without MKE (304 μg protein/ml) for 10 d, then the cells were harvested from the culture and were treated with anti-human FITC-CD34, PE-CD41 and PC5-CD45 MoAb. The expression of the surface antigen was analyzed using a flow cytometer. Values are derived from flow cytometer analysis, as shown in Fig. 4.

ploidy classes, than those of bone marrow or peripheral blood.[50,51] Also, other studies[52—54] have shown that TPO alone does not stimulate maturation and platelet release from megakaryocytes, and that another factor is needed for platelet production. Therefore, the value of the factor that acts on the maturity of megakaryocytes is clearly very high.

MKE contains CPA of CFU-GM from murine bone marrow in serum-free cultures stimulated by IL-3 and Epo, and acts synergistically with G-CSF, M-CSF, IL-1, IL-3 and IL-11.[6] MKE itself does not stimulate the colony formation of CFU-GM.[6] Also, MKE contains regulators that suppress the growth of murine mast cells and histamine synthesis,[7] and we suggested that the mast cell inhibitory factor(s) may be

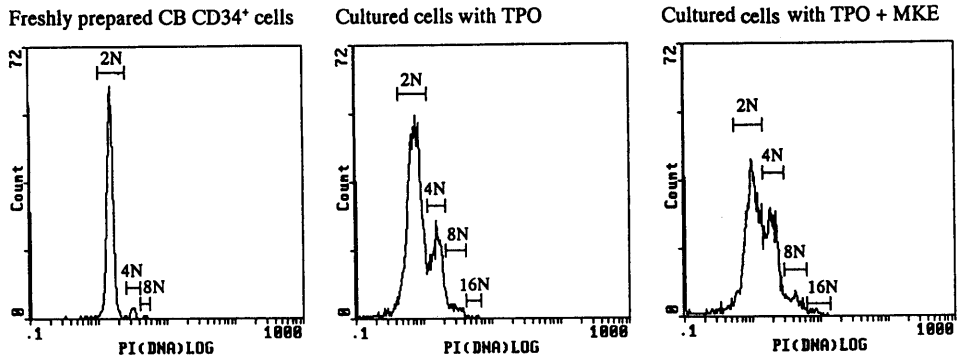

Fig. 4. DNA Poidy Distributions of CD41$^+$ Cells Generated with TPO Alone or TPO+MKE

Purified CB CD34$^+$ cells (1×10^3/ml) were cultured in a plasma clot with or without MKE (304 μg protein/ml) for 10 d, then the cells were harvested from the culture and were treated with anti-human FITC-CD41 MoAb and PI. DNA ploidy distributions were analyzed using a flow cytometer.

Table 5. Effect of Trypsinized and Heated MKE on the Growth of Immature and Mature CFU-Meg

MKE (μg protein/ml)	Number of colonies/1×10^3 CB CD34$^+$ cells			Ratio
	Immature CFU-Meg	Mature CFU-Meg	Total	
Without MKE	37.8±12.0	27.2±4.4	65.0±12.4	1.00
Untreated	25.0±10.1	55.0±7.8	80.0±15.2	1.23
Trypsinized	30.5±6.2	29.8±6.9	60.3±12.2	0.93*
56 °C, 30 min	24.4±6.6	65.6±12.6	90.0±15.2	1.38
70 °C, 30 min	34.4±7.2	48.9±10.9	83.3±15.5	1.28
100 °C, 2 min	26.1±8.5	39.4±10.4	65.6±15.3	1.01*

Plasma clot cultures were prepared in the presence of 50 ng/ml TPO with the 304 μg protein/ml MKE for 10 d, and started with purified CB CD34$^+$ cells (1×10^3/ml). Numbers represent the mean±S.D. of 6 wells from 2 separate experiments. *, $p<0.05$.

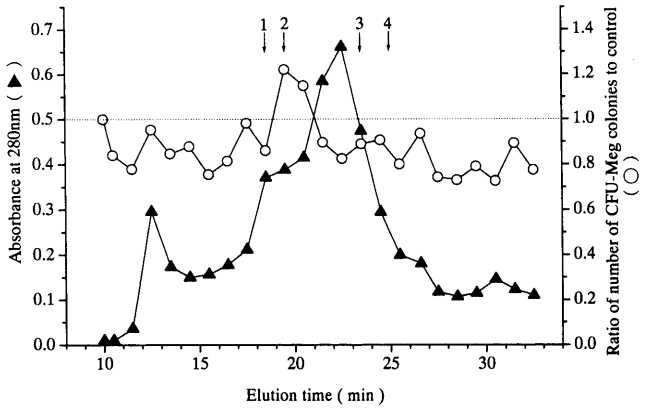

Fig. 5. Elution Profile of MKE on TSKgel-G3000SW$_{XL}$

Sample, MKE 100 μl (1.52 mg); flow rate, 30 ml/h; fractionation, 0.5 ml/fraction. Each number in this figure indicates the elution position of the standard protein for calibration. 1, BSA (68 kDa); 2, albumin from egg (45 kDa); 3, chymotrypsinogen (25 kDa); 4, cytochrome C (12.5 kDa).

different from the colony promoting factor(s), and that MKE may contain different regulators for hematopoiesis, proliferation and differentiation of CFU-GM and mast cells. In this study, the factor of CPA for human CFU-Meg was a heat stable protein, acting to the same extent as the factor of CPA for murine CFU-GM.[6] Moreover, the CPAs for murine CFU-GM and for human CFU-Meg were detected in the same fraction, but the mast cell inhibitory activity was eluted in a fraction different from the fractions showing these 2 CPAs. We may, therefore, reasonably conclude that both CPAs were derived from a factor in MKE. In our previous studies,[6,7] CPA in MKE for CFU-GM from partially purified murine bone marrow cells was detected in a culture supplemented with IL-3 and Epo. This study used highly purified CD34$^+$ cells isolated from human umbilical cord blood for the study of human hematopoiesis by MKE. The MKE increased BFU-E colonies, but showed no increase in CFU-GM colonies in the culture with G-CSF, GM-CSF, IL-3, SCF and Epo, which stimulate maximum colony formation. Thus, the CPA by MKE for human CFU-GM was not observed, maybe because of differences in both culture systems, such as cytokine combinations or cell purity. We cannot precisely explain this difference, but our preliminary experiments showed that MKE promoted CFU-Meg growth from murine bone marrow cells in the culture with recombinant mouse TPO alone. MKE resulted in about a 1.9-fold increase in the number of murine megakaryocyte colonies with 304 μg of MKE (data not shown), indicating a higher promotion in murine megakaryopoiesis than in humans. These results suggest that the colony promoting factor of MKE acts as a hematopoietic regulator for myeloid lineage beyond species specificity, and that the action of MKE depends on the cytokines.

The kidney has a role in the immune system,[35] and produces cytokines such as IL-6 and Epo.[36,37] However, our previous study suggested that the colony promoting factor(s) in MKE does not contain G-CSF, GM-CSF, M-CSF, IL-1, IL-3, IL-4, IL-6, IL-11, SCF or Epo, judging from the biological and chemical properties, the target cells, and the mechanism of action of factor in MKE.[6] In general, murine hematopoietic growth factors have little effect on human hematopoietic stem/progenitor cells *in vitro* due to large differences in their molecular homology. Thus, the species specificity of the colony promoting factor in MKE may be low. Another expla-

nation of the CPA of MKE is that cell-extracellular matrix components, such as fibronectin, collagen, hepalan sulfate, and their relationships, are important in regulating hematopoiesis *in vitro*.[55—57] Especially, the proliferation and maturation of megakaryocyte progenitor cells are influenced by these components,[58] and approximately 30% of CFU-Meg adhere to the extracellular matrix components. However, CPA of MKE does not depend on glycoproteins or glycopeptides, because it is resistant to dithiothreitol and periodate treatment.[6] Furthermore, MKE did not promote the growth of the stromal layer during the culture period in this study (data not shown). From these results we conclude that the CPA of MKE for human CFU-Meg is not a cell-extracellular matrix component and is not derived from the stromal layer. More detailed studies are needed to analyze the active factor(s) in MKE, as well as the influence on and mechanism of the factor(s) in hematopoiesis.

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