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Tumor Necrosis Factor-α Stimulates Colony Formation by a Megakaryoblastic Leukemia Cell Line, CMK

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The CMK cell line is an acute megakaryoblastic leukemia cell line established from a patient with Down's syndrome, and is known to possess characteristics of normal megakaryocytes. Several cytokines with the ability to stimulate megakaryopoiesis, such as interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF), stimulated colony formation by CMK cells. The present study revealed that tumor necrosis factor-α (TNF-α) stimulated colony formation by CMK cells; the potency was almost equal to that of IL-3, IL-6 or GM-CSF. Scatchard plot analysis revealed that CMK cells possess two types of specific binding sites for TNF-α. The high-affinity binding sites had an affinity constant of 0.18 n*M*, and numbered 5,000. The low-affinity binding sites had an affinity constant of 1.8 n*M* and numbered 19,000. These results raise the possibility that TNF-α can act as a growth-stimulating agent on megakaryocyte-lineage cell line.

Key words: TNF-α — Leukemia — Megakaryopoiesis

It is well established that megakaryopoiesis is regulated by two types of growth/differentiation factors, megakaryocyte colony-stimulating factor (Meg-CSF[5]) and thrombopoietin.[1)] The former stimulates the growth of progenitor cells in the cell lineage of megakaryopoiesis, and the latter, also called megakaryocyte potentiator, stimulates the differentiation of megakaryocytes to platelets. Although several cytokines are now known to possess such activity, specific Meg-CSF and thrombopoietin have not yet been identified in spite of many efforts to purify them. One of the reasons why these factors are not yet characterized is the lack of a reliable and simple assay method for screening the biological activity of these factors.

An acute megakaryoblastic leukemia cell line, CMK,[2)] was established from tumor cells of a Down's syndrome patient with acute megakaryoblastic leukemia. These cells express glycoprotein IIb/IIIa, one of the megakaryocyte-specific antigens. Their growth is dependent on cytokines with the ability to stimulate megakaryocyte growth or differentiation, such as interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF)[3)] and interleukin-6 (IL-6).[4)] In contrast, interleukin-1α (IL-1α), interleukin-1β (IL-1β), M-CSF and G-CSF, which have no megakaryocyte growth-stimulating activity, do not stimulate CMK colony formation. These results indicate that CMK cells might share some characteristics with normal megakaryocytes.

In the present study, we found that tumor necrosis factor-α (TNF-α) stimulated colony formation by CMK cells. Specific binding sites for TNF-α on CMK cells were also characterized.

MATERIALS AND METHODS

Hematopoietic factors and antibodies The hematopoietic factors and their antibodies used in the present study were as follows: recombinant human TNF-α with specific activity of 2.3×10^6 U/mg (Asahi Chemical Industry Co., Ltd., Tokyo), recombinant human IL-3 with specific activity of 8.5×10^8 U/mg (Kirin Brewery Co., Ltd., Maebashi), recombinant human GM-CSF with specific activity of 1.0×10^9 U/mg (Kirin Brewery Co., Ltd.), recombinant human IL-6 with specific activity of 6×10^6 U/mg (Ajinomoto Co., Inc., Kawasaki), rabbit anti-recombinant human IL-3 antiserum (Kirin Brewery Co., Ltd.), murine anti-recombinant human IL-6 monoclonal antibody (Ajinomoto Co., Inc.) and murine anti-recombinant human TNF-α monoclonal antibody (Tonen Co., Iruma-gun, Saitama). Rabbit anti-recombinant human GM-CSF antiserum was purchased from Calbiochem-Behring (La Jolla, CA). Neutralizing activities of these antibodies have been reported elsewhere.

Cell lines CMK cells, an acute megakaryoblastic leukemia cell line, was established from a patient with Down's syndrome. The cells were maintained in RPMI 1640 culture medium (Nissui Pharmaceutical Co., Ltd.,

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[5] Abbreviations: Meg-CSF, megakaryocyte colony-stimulating factor; IL-3, interleukin-3; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-6, interleukin-6; IL-1α, interleukin-1α; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; FBS, fetal bovine serum; K_d, dissociation constant; ELISA, enzyme-linked immunosorbent assay.

Tokyo) with 10% fetal bovine serum (FBS; Biocell Laboratories, Carson City, NV), penicillin (100 units/ml) (GIBCO Laboratories, Grand Island, NY), streptomycin (100 μg/ml) (GIBCO), and 2 mM L-glutamine (Nissui Pharmaceutical Co., Ltd.). One-half of the spent medium was replaced with fresh medium every two days.

Colony assay CMK cells were suspended in RPMI 1640 medium containing 0.3% agar (Difco Laboratories, Detroit, MI) and 20% FBS. The cytokines examined were dissolved in the same culture medium and CMK cells were suspended in the solution. One milliliter of agar medium containing 2,000 CMK cells in the presence or absence of cytokines was cultured in a 35-mm tissue culture dish at 37°C in a 5% CO_2 humidified atmosphere for 14 days. The colonies with a diameter of more than 0.5 mm were counted in quadruplicate under an inverted microscope.

Antibodies against various cytokines were added to the colony assay system to determine the actual effect of these cytokines. Normal rabbit serum was used as a control in these neutralization tests.

Radioreceptor assay for TNF-α CMK cells (1×10^6 cells) were suspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin (BSA, Daiichi Pure Chemicals Co., Ltd., Tokyo) and 10 mM HEPES buffer (pH 7.2) and incubated with increasing concentrations of ^{125}I-TNF-α (specific activity, 30 TBq/mmol; Amersham International, Bucks., UK) in the presence or absence of a 1,000-fold excess of unlabeled TNF-α at a total volume of 0.5 ml at 37°C for 60 min. After incubation, CMK cells were washed three times with cold PBS(−) (Nissui Pharmaceutical Co., Ltd.) and radioactivity was counted. Specific binding was calculated as total binding minus non-specific binding; the latter was determined in the presence of a 1,000-fold excess of unlabeled TNF-α. The dissociation constant (K_d) and the number of receptors were calculated by Scatchard plot analysis.

ELISA for IL-6 and GM-CSF Immunoreactive IL-6 and GM-CSF in culture media conditioned by TNF-α-stimulated CMK cells were measured by enzyme-linked immunosorbent assay (ELISA). CMK cells (2×10^5/ml) were suspended in 10 ml of RPMI medium containing 10% FBS and incubated for 72 h in the presence or absence of TNF-α at concentrations ranging from 0.01 to 100 ng/ml. The resultant supernatant was collected and stored at −20°C. IL-6 was measured with an ELISA kit for human IL-6 (Quantikine™, R and D Systems, Minneapolis, MN) and GM-CSF was measured with an ELISA kit for human GM-CSF (Factor-Test™, Genzyme Corp., Boston, MA).

Statistical analysis All data obtained by colony assay were from quadruplicate tests and were statistically analyzed by using Student's t test.

RESULTS

Colony assay The effects of the various cytokines on colony formation by CMK cells are shown in Fig. 1. TNF-α significantly stimulated CMK-colony formation at doses greater than 1.0 ng/ml in a dose-dependent manner. IL-3, GM-CSF and IL-6 also stimulated colony formation by CMK cells (Fig. 1). There was no difference between colonies stimulated by each cytokine in size and morphology. In this experiment the maximal numbers of colonies stimulated by TNF-α, GM-CSF, IL-3 and IL-6 at the optimal dose were 128±16.2, 142±6.4, 106±1.4 and 98±7.1, respectively. On the basis of maximal number of colonies, the activity of TNF-α was statistically significantly greater than that of IL-3 or IL-6, but not GM-CSF.

Effects of antibodies Anti-TNF-α antibody at a concentration of 10 μg/ml or more completely suppressed TNF-α-stimulated CMK colony formation, when the dose of TNF-α was 10 ng/ml (Fig. 2). The neutralizing effect of anti-TNF-α antibody is specific for TNF-α, since this antibody did not inhibit IL-3-, IL-6- or GM-CSF-stimulated colony formation (Fig. 3). The same experiments were performed with the other three antibodies, and it was found that their neutralizing effect was specific for the respective cytokines when they were used at the following concentrations: anti-IL-3 antiserum at a dilution of 1:1,000, anti-GM-CSF antibody at a concentra-

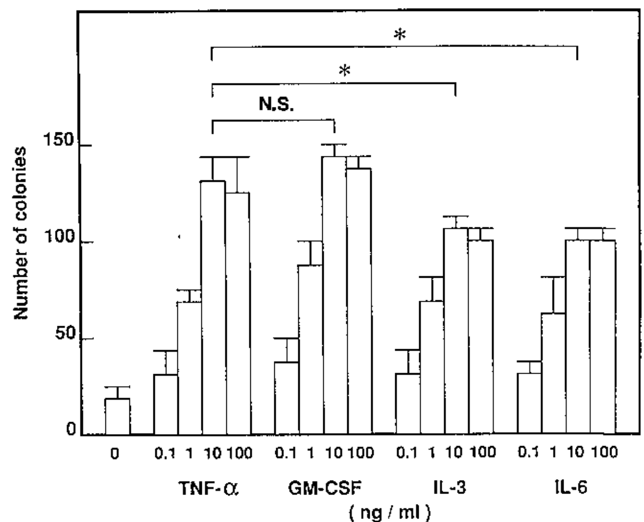

Fig. 1. CMK colony-stimulating activity of TNF-α, GM-CSF, IL-3 and IL-6. Each cytokine was used at doses of 0.1, 1, 10 and 100 ng/ml. Colony assay was performed in quadruplicate. Each column represents the mean±SD. *, $P<0.05$; N.S., not significant.

tion of 40 μg/ml, anti-IL-6 monoclonal antibody at a concentration of 20 μg/ml. Moreover, antibodies against IL-3, IL-6 and GM-CSF at the above doses had no inhibitory effect on colony formation stimulated by TNF-α at a dose of 10 ng/ml (Fig. 4). Normal rabbit serum at a dilution of 1:100 had no effect on colony formation in the presence or absence of cytokines.

Radioreceptor assay for TNF-α The saturation isotherm of specific binding of ^{125}I-TNF-α to CMK cells are shown in Fig. 5. CMK cells were found to have specific binding sites for TNF-α. Scatchard plot analysis revealed two types of binding sites; high affinity with the K_d of 0.18 nM and low affinity with K_d of 1.8 nM. The numbers of these binding sites per cell were 5,000 and 19,000, respectively.

ELISA Immunoreactive GM-CSF and IL-6 in culture media conditioned by CMK and MT-2 cells (human T-cell leukemia virus type I-infected T-cell line)[5] were measured by ELISA. The detection limit of IL-6 by this ELISA was 31 pg/ml. The concentration of IL-6 in culture media conditioned by MT-2 cells, which are known to hyperproduce IL-6, was 26,000 pg/ml. Immunoreactive IL-6 was undetectable (less than 31 pg/ml) in the culture media conditioned by TNF-α-stimulated CMK cells. The detection limit of GM-CSF was 8 pg/ml.

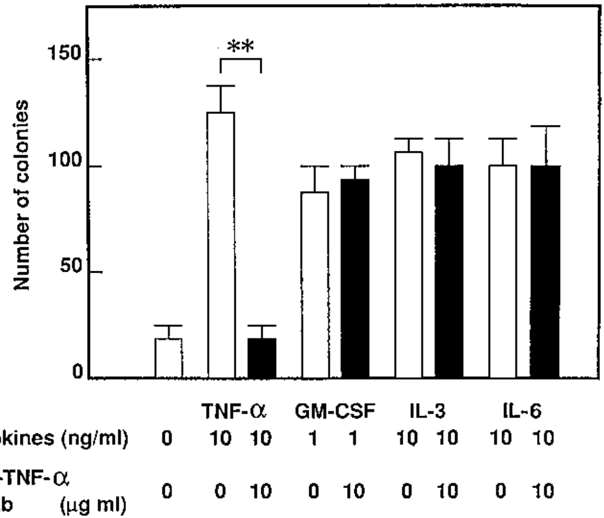

Fig. 3. Effect of anti-TNF-α monoclonal antibody on CMK colony formation stimulated by TNF-α, IL-3, GM-CSF and IL-6. Colony assay was performed in quadruplicate. Each column represents the mean±SD. CMK colony formation was stimulated by 10 ng/ml TNF-α, 10 ng/ml IL-3, 1 ng/ml GM-CSF and 10 ng/ml IL-6. RPMI 1640 medium was used for the negative control. □: cultures with normal rabbit serum at ×100 dilution. ■: cultures with anti-TNF-α monoclonal antibody at 10 μg/ml. **, $P<0.01$.

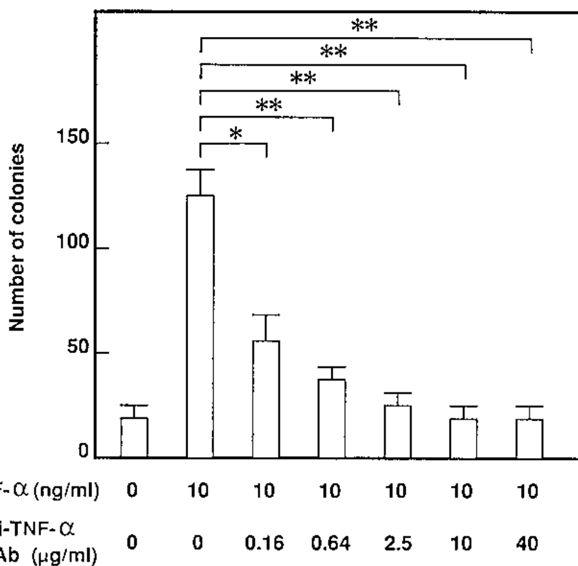

Fig. 2. Effect of anti-TNF-α monoclonal antibody on CMK colony formation stimulated by 10 ng/ml TNF-α. Colony assay was performed in quadruplicate. Each column represents the mean±SD. Anti-TNF-α monoclonal antibody was preincubated with TNF-α for 60 min at 37°C. RPMI 1640 medium was used for the negative control. Normal rabbit serum was used as the control antibody in the absence of anti-TNF-α monoclonal antibody. **, $P<0.01$; *, $P<0.05$.

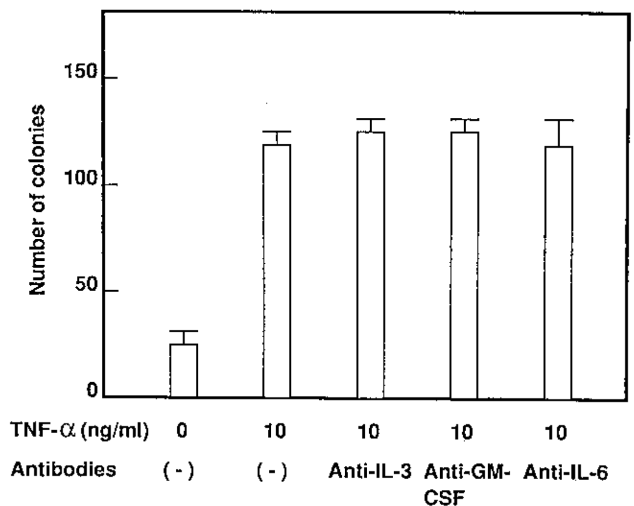

Fig. 4. Effect of anti-IL-3 antiserum, anti-GM-CSF antibody and anti-IL-6 antibody on colony formation by TNF-α-stimulated CMK cells. Colony assay was performed in quadruplicate. Each column represents the mean±SD. The antibodies were used at the following doses: anti-IL-3 antiserum, ×1,000 dilution; anti-GM-CSF antibody, 40 μg/ml; anti-IL-6 antibody, 20 μg/ml. Normal rabbit serum was used as the control antibody at ×100 dilution. TNF-α was used at a dose of 10 ng/ml. RPMI 1640 medium was used for the negative control.

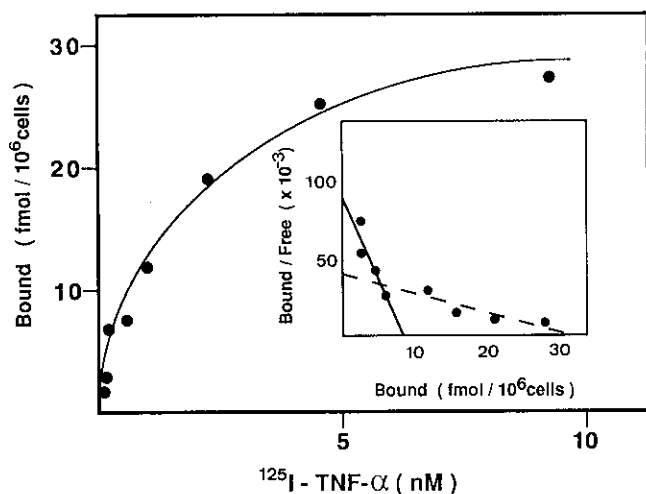

Fig. 5. Specific binding of ^{125}I-TNF-*α* to CMK cells. Each point represents the mean of duplicated assays. The inset shows the data obtained by Scatchard plot analysis.

The concentration of GM-CSF in culture media conditioned by MT-2 cells, which are known to hyperproduce GM-CSF, was 400 pg/ml. Immunoreactive GM-CSF was undetectable (less than 8 pg/ml) in the culture media conditioned by TNF-*α*-stimulated CMK cells.

DISCUSSION

This study showed that TNF-*α* stimulated colony formation by CMK cells; the potency of TNF-*α* estimated in terms of the dose required for the formation of 50 colonies was almost equal to that of GM-CSF, IL-3 or IL-6. With regard to maximal number of colonies, it was more potent than IL-3 and IL-6. These results suggest that TNF-*α* is a potent CMK growth-stimulating agent.

It is well established that TNF-*α* possesses potent cytotoxic activity against various cancer cells *in vitro*. Conversely, recent studies have shown that TNF-*α* stimulates cellular proliferation, determined by cell number counting and thymidine incorporation assay, in a human fibroblast cell line (FS-4),[6] human normal T lymphocytes,[7] a chronic lymphocytic leukemia cell line[8] and a hairy cell leukemia cell line.[9] In the present study, TNF-*α* stimulated CMK colony formation at a concentration of 10 ng/ml or more. Previous studies have demonstrated that proliferation of the above cells was stimulated by TNF-*α* at a dose of 10 ng/ml or more. Alternatively, the cytotoxic activity of TNF-*α* was reported to be elicited at doses ranging from 10 to 40 ng/ml in epithelial[10] and hematopoietic cells.[11-13] Taking these findings together, it is reasonable to postulate that the effective dose of TNF-*α* for CMK cells *in vitro* is comparable with that for other TNF-*α*-responsive cells, for either stimulatory or inhibitory effects.[14]

It is likely that TNF-*α* stimulation of CMK colony formation is mediated by TNF-*α* receptors on CMK cells. The present study revealed that CMK cells possess two types of specific binding sites for TNF-*α*. The high-affinity binding sites of CMK cells have an affinity constant of 0.18 n*M*, slightly lower than the affinity constant of the receptor expressed in myeloid leukemia cell lines including HL-60, U-937, KG-1 and K-562, as reported by Munker *et al.*[15] The number of binding sites with high affinity on CMK cells was greater than that on myeloid leukemia cell lines. With regard to low-affinity binding sites, Imamura *et al.*[16] demonstrated two types of specific binding sites for TNF-*α* on human monocytes. The low-affinity binding sites on our CMK cells had a lower affinity constant but were more numerous than those expressed on monocytes. Recently, two types of possible TNF-*α* receptors have been purified,[17, 18] sequenced[19] and cloned.[20-22] The relation between these two types of receptors and the two binding sites with different affinity on CMK cells has not yet been elucidated. In any case, it is worth noting that there is no report of TNF-*α* receptors expressed on cells belonging to the megakaryocyte lineage.

With the aim of clarifying the mechanism responsible for the proliferative effect of TNF-*α* on CMK cells, we have tested the possibility that TNF-*α* stimulates the release of cytokines including GM-CSF and IL-6 from CMK cells in an autocrine fashion.[23, 24] Since TNF-*α* did not stimulate the release of GM-CSF or IL-6, which have the ability to potentiate CMK growth, it seems reasonable to speculate that the TNF-*α*-associated signal transduction pathway directly activates the intracellular machinery responsible for CMK cell growth. However, the possibility that release of unknown cytokines with ability to stimulate CMK growth was induced by TNF-*α* cannot be ruled out.

Clinically, the fact that TNF-*α* stimulated the growth of CMK cells, a cell line belonging to the megakaryocyte lineage, *in vitro* should be carefully evaluated. A recent study by Caux *et al.*[25] demonstrated that TNF-*α* potentiates IL-3- or GM-CSF-stimulated cellular growth of human $CD34^+$ hematopoietic cells, possible stem cells. Moreover, our study demonstrated that CMK colony-stimulating activity of TNF-*α* was promoted with the addition of IL-1*α*, IL-1*β* or M-CSF, although these cytokines alone did not stimulate the growth of CMK cells (unpublished data). This observation raises the possibility that TNF-*α* might be one of the cytokines related to thrombopoiesis. However, in a recent phase I clinical trial of TNF-*α* for cancer therapy,[26] thrombocytopenia was noted as an adverse effect. Further studies are

required to determine whether TNF-α has a specific effect on highly purified human megakaryocyte-lineage cells. At any rate, we verified that TNF-α stimulated the growth of CMK cells in a direct manner; this cell line could be useful as a model for research on signal transduction related to TNF-α-modulated cellular growth.

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Addendum: After the submission of this manuscript, Miura *et al.* reported by means of tritiated thymidine incorporation assay that TNF-α stimulated the growth of CMK cells.[27]

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