RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 928–931, October 1989

# Secretion of Transforming Growth Factor-$\beta$ by Human Myelogenous Leukemic Cells and Its Possible Role in Proliferation of the Leukemic Cells

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**Transforming growth factor(TGF)-$\beta$ activity was found in the neutral extracts of human myelogenous leukemic cells or K562 cells and the conditioned medium from K562 cell culture. BALB/c 3T3 cells grown in soft agar in the presence of TGF-$\beta_1$ produced an activity that stimulated the growth of K562 cells. This activity was non-dialyzable, acid-stable, heat-sensitive and partially inactivated by pronase treatment. These results suggest a mutual growth reliance between the leukemic cells and fibroblasts mediated by paracrine growth factors produced by these cells.**

Key words: Human myelogenous leukemic cell — Fibroblast — Transforming growth factor-$\beta$ — Leukemic cell growth factor

Hematopoietic stem cells have been shown to proliferate and differentiate under the control of various hemopoietic growth factors[1] and microenvironments[2] in the bone marrow, especially stromal cells. Even leukemic cells have been shown to grow under the influence of the stromal cells.[3] We have already found that the extract of K562 cells, a human leukemic cell line, potentiated the growth of BALB/c 3T3 cells.[4] So we examined in the present study the possibility of mutual growth reliance between leukemic cells and fibroblasts.

BALB/c 3T3, clone A31 (3T3 cells) and NRK-49F (NRK cells), a subline of normal rat kidney fibroblasts (Dainippon Pharmaceutical Co., Osaka) were cultured in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS). Human fetal lung-derived fibroblasts (WI-38, kindly supplied by the Japanese Cancer Research Resources Bank-Cell) were maintained in the same medium with 10% FCS. K562, a clonal cell line derived from the leukemic cells at the blast crisis of human chronic myelogenous leukemia (CML BC), was maintained in RPMI 1640 supplemented with 10% FCS. Peripheral blood was obtained from a patient with acute myeloblastic leukemia (AML; classified as M1 according to the FAB criteria) and a patient with CML BC. Leukocytosis ($24\times10^9$/liter with 76% blasts in the AML patient and $98\times10^9$/liter with 61% blasts in the CML BC patient) was found at diagnosis. Mononuclear cells were isolated by Ficoll-metrizoate centrifugation. The extract of leukemic cells with PBS (phosphate-buffered saline, pH 7.4) and the conditioned medium (CM) from K562 cells were prepared as reported previously.[4] In some experiments, K562 CM was acidified and neutralized according to the reported method.[5] Transforming growth factor (TGF)-$\beta$ activity was measured in terms of the potential to promote colony formation of NRK cells in the presence of epidermal growth factor (EGF: 0.1 ng/ml). One ml of 0.6% agar containing McCoy's 5A medium supplemented with 10% calf serum (CS) was added to a 35 mm culture dish as the base layer, on which 1 ml of 0.3% agar containing test samples and indicator cells (NRK, $1\times10^3$ cells) in the same medium was layered and cultured for 10 days. The number of resultant NRK colonies was counted. The growth-promoting activity on K562 cells was measured as the ability to stimulate the colony formation of K562 cells ($1\times10^3$ cells) in 0.33% soft agar containing RPMI 1640 medium with 2.75% FCS essentially according to the method of Perkins *et al.*[6] K562 cells formed colonies with less than 1% plating efficiency in the absence of test samples. The number of K562 cell colonies was measured on the 10th day.

PBS extracts of leukemic cells from patients with AML or CML BC, and PBS extract of K562 cells were found to have activities to potentiate NRK colony formation in the presence of EGF. The K562 CM also demonstrated a similar activity which was almost completely neutralized by anti TGF-$\beta_1$ IgG (R&D Systems, Inc., Minneapolis, MN) raised against porcine platelet TGF-$\beta_1$ (Table I). This antibody is cross-reactive with human TGF-$\beta_1$ and TGF-$\beta_2$. The activity in the CM amounted almost to 56% of the activity recovered from the same CM previously acidified and neutralized. In this table, the number of NRK colonies formed in the presence of EGF without cell extract or CM was considerably larger than that reported by Roberts *et al.*[7] The reason for this discrepancy is not clear, but it may have resulted from the difference in the culture conditions for maintaining NRK cells. These results suggest that a

Table I. Stimulation of NRK Colony Formation by Extracts of and CM from Human Leukemic Cells

| Cell extract or conditioned medium | NRK colonies | |
|---|---|---|
| | −EGF | +EGF |
| None | 0 | 96±7 |
| AML extract | 0 | 194±17 |
| CML BC extract | 0 | 209±16 |
| K562 extract | 21±3 | 268±45 |
| K562 CM | 15±4 | 305±9 |
| K562 CM+Ab[a] | 2±1 | 114±7[d] |
| K562 CM+n-IgG[a] | 13±2 | 282±25[e] |
| K562 CM[b] | 4±2 | 449±52 |
| TGF-$\beta_1$, 1 ng/ml[c] | 2±1 | 149±14 |
| TGF-$\beta_1$, 5 ng/ml[c] | 1±0 | 331±21 |

PBS extracts of leukemic cells (30 $\mu$g protein/ml) or 50 $\mu$l of 30 times concentrated K562 CM ($8\times10^5$ K562 cells/ml for 2 days) was added to soft agar with or without EGF (0.1 ng/ml). The resultant NRK colonies were counted at 10 days. CM, conditioned medium; Ab, anti TGF-$\beta_1$ rabbit IgG; n-IgG, IgG from non-immunized rabbit serum.

*a*) Thirty times concentrated K562 CM (50 $\mu$l) was pre-incubated for 1 h at room temperature with 40 $\mu$g of anti TGF-$\beta_1$ rabbit IgG or the same amount of IgG from non-immunized rabbit serum. Under these conditions the antibody is sufficient to neutralize the activity of about 4 ng of TGF-$\beta$ according to the manufacturer's instructions.

*b*) K562 CM previously acidified and neutralized.

*c*) Concentration of TGF-$\beta_1$ in soft agar.

*d*) $P<0.001$ vs. K562 CM+n-IgG by Student's $t$ test.

*e*) Not significant vs. K562 CM alone.

Values represent means±SD of 4 determinations.

considerable amount of TGF-$\beta$ was secreted from the leukemic cells as an active form. The much lesser extent of secretion of active TGF-$\beta$ from a variety of cultured cell lines other than leukemic cells has been reported by other investigators.[5] Elevated levels of TGF-$\beta$ activity in serum of CML patients have been reported.[8]

When TGF-$\beta_1$ (0.5–10 ng/ml, Wako Pure Chemical Industries, Ltd., Tokyo) was added to agar medium, neither stimulation nor inhibition of K562 colony formation was observed (data not shown). However, the addition of TGF-$\beta_1$ in agar medium greatly stimulated the colony formation of 3T3 cells. 3T3 cells ($2\times10^3$ cells) seeded in soft agar containing 1.25 and 10 ng/ml TGF-$\beta_1$ yielded 30±10 and 481±34 colonies, respectively. Therefore, it is probable that the secreted TGF-$\beta$ promotes the growth of fibroblasts which may result in the fibrosis of the bone marrow often seen in the course of CML. The fibrosis is rarely associated with AML. This may be partly because of the fact that the blast cell count is usually higher in CML BC than in AML. TGF-$\beta$, when subcutaneously injected in newborn mice, was shown to induce fibrosis and angiogenesis.[9] TGF-$\beta$ was reported to be a potent chemoattractant for fibroblasts[10] and to promote the synthesis of collagen.[9] It was also shown to stimulate the proliferation of fibroblasts cultured *in vitro*, such as human foreskin fibroblasts, 3T3 cells and mouse embryo-derived fibroblasts (AKR-2B), although it was inhibitory to the growth of a variety of cultured cells.[11]

Bone marrow stromal cells were reported to support the growth of a variety of tumor cells.[12] It is possible, therefore, that TGF-$\beta$ secreted from the leukemic cells might stimulate the growth of fibroblasts in the bone marrow which in turn influences the growth of leukemic cells. To test this possibility, we first examined the effect of the extract of soft agar containing 3T3 colonies formed by the addition of TGF-$\beta_1$ on the growth of K562 cells. As shown in Fig. 1, the 3T3 colony extract stimulated the colony formation of K562 cells in a dose-dependent manner. The slight increase in the number of K562 colonies in two controls is probably due to CS remaining in the agar extract. Second, we examined the effect of CM from 3T3 monolayer culture on the growth of K562 cells. The 3T3 CM also stimulated the K562 colony formation (Fig. 2).

The above results were obtained by using murine fibroblasts, 3T3 cells. Therefore, we tested whether or not the growth of human leukemic cells depends on human

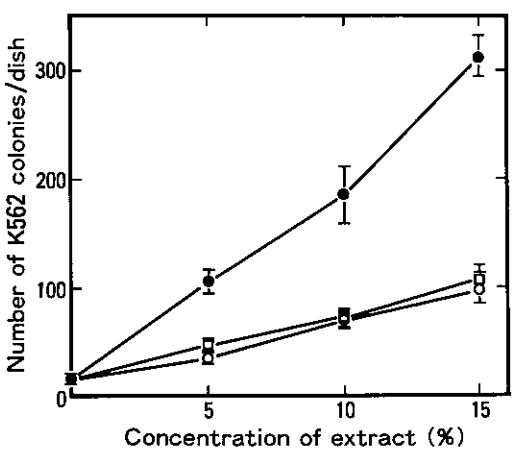


Fig. 1. Effect of the extract of soft agar containing 3T3 cell colonies on K562 cell growth. 3T3 cells ($2\times10^3$) were seeded in soft agar containing 10 ng/ml TGF-$\beta_1$ and cultured for 10 days. The soft agar with the resultant 3T3 cell colonies was frozen, thawed and centrifuged at 15,000 rpm for 30 min. The supernatant (●) was tested for its activity to promote the growth of K562 cells in soft agar. □: the supernatant (extract) of soft agar seeded with 3T3 cells, but containing no TGF-$\beta_1$. ○: the extract of soft agar containing TGF-$\beta_1$, but without 3T3 cell seeding. Means±SD of 4 determinations.

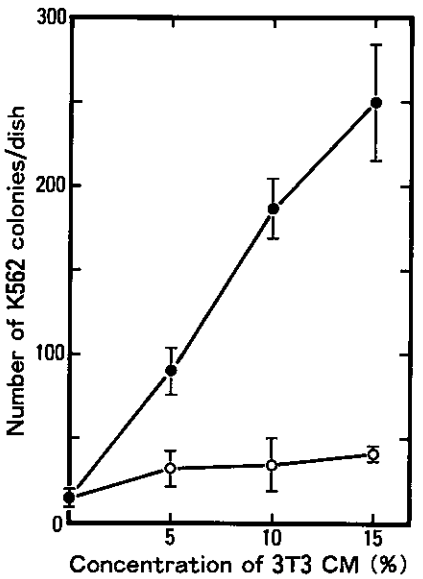


Fig. 2. Effect of CM from 3T3 monolayer culture on K562 cell growth in soft agar. 3T3 cells were cultured and grown to the late log phase ($3–4\times10^4$ cells/cm$^2$). The medium was changed to MEM containing 1% FCS and the CM was obtained after incubating the cells for 24 h. Increasing concentrations of 3T3 CM (●) or the control medium (MEM+1% FCS) (○) were tested for their abilities to support the growth of K562 cells in soft agar. Means±SD of 4 determinations.

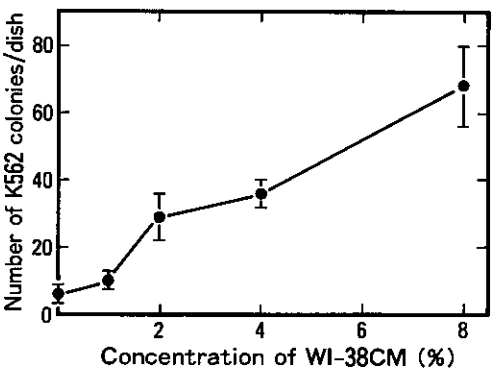


Fig. 3. Effect of CM from WI-38 monolayer culture on K562 cell growth in soft agar. WI-38 cells were cultured in MEM containing 10% FCS and grown to the late log phase ($3–4\times10^4$ cells/cm$^2$). The culture medium was then refreshed and the CM was obtained after incubating the cells for 2 days. Increasing amounts of the CM were added to the soft agar of the assay system and tested for their activities to promote the growth of K562 cells in soft agar. An appropriate amount of FCS was added to adjust its final concentration in the assay agar to 2.75%. Means±SD of 4 determinations.

fibroblasts. The K562 colony formation was stimulated by the increasing amounts of CM from WI-38 monolayer culture (Fig. 3). This result suggests that the growth of leukemic cells was promoted by human fibroblasts as well. A similar result was obtained by using stromal cells obtained from human bone marrow (unpublished result).

The growth-promoting activity on K562 cells in 3T3 CM was non-dialyzable, heat-stable at 56°C for 30 min, almost completely inactivated by heat treatment at 100°C for 5 min, partially inactivated by pronase treatment (30 $\mu$g/ml at 37°C for 3 h), stable at pH 2 and adsorbed on concanavalin A agarose. The addition of known growth factors such as G-CSF (0.1–100 ng/ml), GM-CSF (0.1–100 ng/ml), a 1:1 mixture of IL-1$\alpha$ and IL-1$\beta$ (10–100 U/ml), IL-3 (10–200 U/ml), IL-6 (1–1000 U/ml) and EGF (0.2–10 ng/ml) did not stimulate the colony formation of K562 cells in soft agar. Therefore, these growth factors were not likely to be candidates for the activity (data not shown). The growth-promoting activity on K562 cells in WI-38 CM showed almost the same properties as described above and was not neutralized with polyclonal antibodies against human G-CSF and GM-CSF. Purification of the active entity is necessary to establish whether or not this activity is due to a new molecule.

In summary, human myelogenous leukemic cells appear to secrete active TGF-$\beta$ and stimulate fibroblasts to produce the growth factor which in turn promotes the leukemic cell growth, creating a paracrine loop in favor of tumor cell growth. The possibility of mutual growth reliance between leukemic cells and stromal cells has been suggested by Griffin *et al.*[13] They reported that the leukemic cells from 10 of 17 AML patients secreted IL-1, which stimulated endothelial cells to produce GM-CSF and G-CSF. This finding together with the results reported here may imply a tumor and host cell interaction in favor of tumor progression.

We are grateful to Miss M. Shimosaka for expert technical assistance.

*(Received May 31, 1989/Accepted September 2, 1989)*

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