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Stromal Cell-dependent Growth of Leukemic Cells from Murine Erythroblastic Leukemia

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Transplantable erythroblastic leukemia was induced by 300-rad irradiation of C3H mice. Conditions for *in vitro* growth of the leukemic cells were studied. None of interleukin-3, granulocyte/macrophage colony-stimulating factor and erythropoietin could support the growth of the cells *in vitro*. In contrast, the leukemic cells grew into a stroma-dependent cell line, ELM-D, in close contact with the stromal cell layer of 900-rad-irradiated long-term bone marrow culture. A stroma-independent cell line, termed ELM-I-1, was further established from the non-adherent population in the co-culture of the leukemic cells, ELM-D, with stromal cells. Reverse transcriptase activity was not detectable in ELM-D or ELM-I-1 cells. Studies on binding and cross-linking of ^{125}I-erythropoietin showed that ELM-I-1 cells had erythropoietin receptors, and two major radiolabeled protein products with molecular weights of 120 kDa and 140 kDa were detected on sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions.

Key words: Hemopoietic microenvironment — Erythroblastic leukemia — Erythropoietin receptor — Stroma-dependent cell line — Erythroid precursor cell

Studies on leukemia *in vivo* have often been complicated by the use of mixed populations of normal and leukemic cells. A number of attempts have been made to establish cell lines which grow *in vitro* to overcome this problem and to clarify the character of the leukemic cells. Some leukemic cells could be adapted to grow in cultures *in vitro*. However, this does not necessarily imply that these cells are independent of the regulatory system of hemopoiesis. A possible approach to determine whether leukemic cells are subject, even in part, to the normal regulatory system of hemopoiesis would be to define the conditions necessary for the leukemic cells to grow *in vitro*.

A transplantable erythroblastic leukemia in C3H mice[1] is available as a model for human leukemia. Reverse transcriptase activity was not detectable in the spleen or the liver of the mice, or in various kinds of human leukemia cells.[2] We have now established transformed erythroid precursor cell lines derived from this leukemia. This paper reports some characteristics of the leukemic cell lines and the conditions for leukemic cell growth *in vitro*.

MATERIALS AND METHODS

Mice and Erythroblastic Leukemia Mice of C3H/HeNSlc strain were purchased from Shizuoka Animal Farm (Shizuoka), housed under specific pathogen-free conditions, and used at 8–10 weeks of age.

Erythroblastic leukemia was induced by irradiation of C3H mouse with 300 rads of X-rays.[1] Spleen cells of the leukemia-bearing mice were transferred to syngeneic mice, and the leukemia has been maintained in our laboratory.

Establishment of Stromal Cell Layer Long-term bone marrow culture was established as described previously.[3,4] Cells from femora and tibiae were flushed into 7 ml of alpha-minimum essential

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medium (α-MEM)*[6] supplemented with 20% horse serum (Nippon Bio-Supply Center, Tokyo), 100 U/ml penicillin, 50 μg/ml streptomycin and $2\times10^{-7}M$ hydrocortisone-succinate in a flask with a base area of 25 cm^2 (Falcon 3013, USA). The cultures were maintained at 37° in an atmosphere of 5% CO_2 in air, and were fed at weekly intervals by replacing half the medium with fresh growth medium. Two weeks later, cultures were washed with growth medium and irradiated with 900 rads of X-rays to deplete hemopoietic cells. These cultures were used for experiments as stromal cell layer.

Preparation of Colony-stimulating Factors (CSFs) Conditioned medium from an interleukin-3 (IL-3)-producing cell line, WEHI-3,[5] was prepared as follows. WEHI-3 cells were incubated at a concentration of 1×10^6 cells/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Medium was harvested after 48 hr of incubation and stored at −20° until use. The conditioned medium (WEHI-3-CM) was used as a source of IL-3.

Murine abdominal wall-conditioned medium (AWCM) was prepared as described previously,[6] and was used as a source of granulocyte/macrophage colony-stimulating factor (GM-CSF).

Erythropoietin Recombinant erythropoietin (r-Epo) was produced by $\phi2$ cells[7] harboring the human Epo gene as described elsewhere.[8]

Leukemic Cell Culture Spleen cells from the mice bearing erythroblastic leukemia were suspended in α-MEM supplemented with 10% FCS at a concentration of 2×10^6 cells/ml, and were cultured in a flask with a base area of 25 cm^2 at 37° in humidified air. To some flasks, either 10% (v/v) WEHI-3-CM, 10% AWCM, or 2 U/ml of r-Epo was added. The cells were also seeded onto the stromal cell layer without WEHI-3-CM, AWCM, and r-Epo. These cultures were fed at weekly intervals as described for stromal cells. Numbers of viable cells were counted every week.

Assay of Reverse Transcriptase and Other DNA Polymerases Reverse transcriptase and other DNA polymerase activities of the cell extract were measured by using phosphocellulose column chromatography as described elsewhere.[1,2,9]

*[6] Abbreviations: α-MEM, alpha-minimum essential medium; IL-3, interleukin-3; FCS, fetal calf serum; WEHI-3-CM, WEHI-3 cells conditioned medium; AWCM, murine abdominal wall-conditioned medium; GM-CSF, granulocyte/macrophage colony stimulating factor; r-Epo, recombinant erythropoietin; RT, reverse transcriptase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; *Kd*, dissociation constant; Epo-R, erythropoietin receptor; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Binding and Cross-linking of Radioactive Erythropoietin to Cells Recombinant-Epo was labeled with ^{125}I by the iodogen method,[8] and was used for binding and cross-linking studies as described elsewhere.[8] The specific radioactivity of ^{125}I-Epo was 40 μCi/μg Epo.

For binding studies, 100 μl of cell suspension (5×10^6 cells) was mixed with 50 μl of Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 60mM Hepes, 0.3% bovine serum albumin (BSA), 0.6% NaN_3, and ^{125}I-Epo in a 1.5 ml tube. After incubation for 1 hr at 37°, the cells were pelleted, washed, re-suspended in 200 μl of PBS and layered on 800 μl of PBS containing 10% BSA. The cells were separated from the unbound radioactive ligand by centrifugation at 400g for 5 min. The tube contents were frozen in solid CO_2/ethanol, and the tips were cut off. The radioactivity of the tips was counted on a γ-counter. Specific binding was calculated by subtraction of non-specific binding (in the presence of 200-fold unlabeled Epo) from total binding (in the absence of unlabeled Epo). The number of receptors and their dissociation constants (*Kd*) were determined by Scatchard analysis.

For covalent binding of ^{125}I-Epo to its cellular receptor, 2×10^7 cells were incubated at 37° for 1 hr in 300 μl of RPMI-1640 containing 2% BSA, 20mM Hepes, 0.2% NaN_3, and 23 ng of ^{125}I-Epo (2×10^6 cpm) in the absence or the presence of unlabeled Epo. After incubation, the mixture was cooled on ice and then the cells were washed three times. The cells were re-suspended in 600 μl of PBS and mixed with 6 μl of disuccinimidyl suberate, a reagent for cross-linking, freshly dissolved in dimethylsulfoxide. The mixture was incubated at 0° for 1 hr, and the cells were washed twice with cold PBS and re-suspended in 80 μl of 20mM Hepes containing 1% Triton X-100, 2mM phenylmethylsulfonyl fluoride, 10μM pepstatin, 10 μM leupeptin, 10μM antipain and 2mM EGTA. After incubation for 30 min at room temperature, insoluble material was removed by centrifugation at 12,000g for 10 min. The supernatant was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis[10] with a separating gel containing 7.5% acrylamide. The gels were dried and subjected to autoradiography at −70° with X-ray films and intensifying screens.

RESULTS

Conditions for *in vitro* Growth of the Leukemic Cells Spleen cells of the leukemic mice died out rapidly in the conventional culture (α-MEM+10% FCS). Almost all cells died within 2 weeks. After 3 weeks of culture, fibroblastic (adherent) cell layers were estab-

Table I. Primary Cultures of the Leukemic Spleen Cells under Various Conditions

Culture condition	Number of cells/ml			
	weeks of culture			
	0[a]	1	3	6
Growth medium (10% FCS)	2×10^6	2.3×10^4	$<10^4$	1.3×10^4 [e]
Growth medium + IL-3[b]	2×10^6	1.8×10^4	$<10^4$	ND[h]
Growth medium + GM-CSF[c]	2×10^6	2.1×10^4	$<10^4$	ND
Growth medium + Epo[d]	2×10^6	2.0×10^4	$<10^4$	ND
Growth medium + stromal cell layer[g]	2×10^6	2.5×10^4	1.5×10^4	5.8×10^5 [f]

a) All cultures were started at a concentration of 2×10^6 leukemic spleen cells/ml.
b) WEHI-3-CM (10% v/v) was added as a source of IL-3.
c) AWCM (10% v/v) was added as a source of GM-CSF.
d) r-Epo (2 U/ml) was added.
e) These cells did not induce leukemia in host mice.
f) After pipetting, non-adherent cells were counted. These cells induced leukemia in host mice.
g) Establishment of stromal cell layer was described in "Materials and Methods."
h) Not counted (very few).

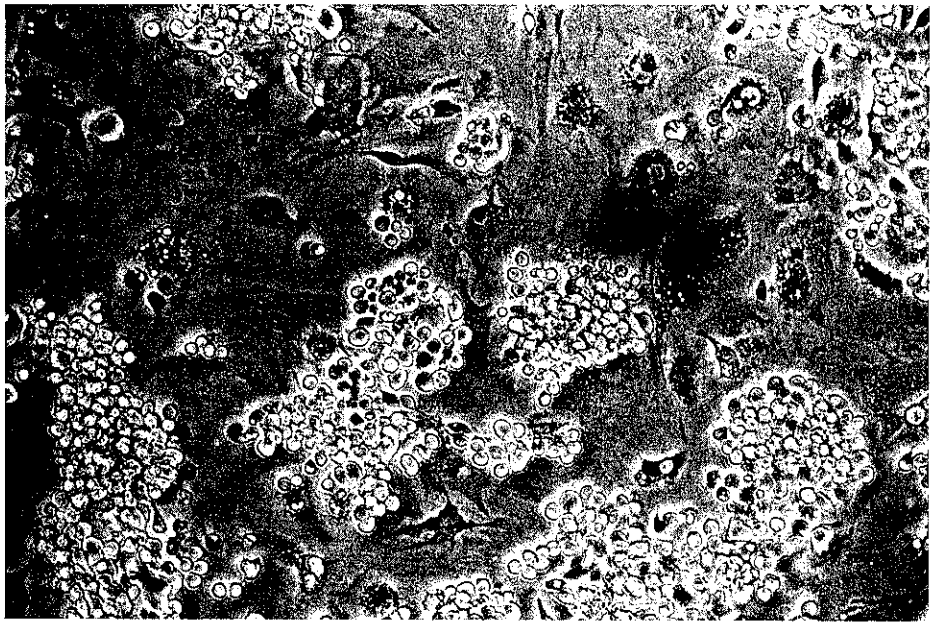

Fig. 1. Phase-contrast photomicrograph. The cells (ELM-D cells) grow on top of the stromal cell layer. (×100)

lished, but these cells did not induce leukemia in host mice. None of IL-3, GM-CSF and Epo could support the growth of the cells *in vitro* (Table I).

The number of viable cells also showed an initial decrease when spleen cells were seeded on top of a stromal cell layer. However, some cells began to grow after 3 weeks of culture. As shown in Fig. 1, the cells grew on top of the stromal cell layer with some cells being detached from the layer. The growth was so rapid that cultures were fed with fresh

medium twice or three times a week after 5 weeks of culture. Non-adherent cells were re-suspended every week in fresh growth medium without IL-3, GM-CSF, Epo or stromal cells. Non-adherent cells from 4–6 weeks of culture did not grow for a long time without stromal cells. However, transfer of the suspended (non-adherent) cells from 7 weeks of culture into new flasks resulted in a stroma-independent growth of leukemic cells. An intravenous injection of the cells induced leukemia in C3H mice. The cells were maintained in α-MEM supplemented with 10% FCS, and a cell line, designated as ELM-I-1, was thus established.

The cultures of leukemic cells which grew on top of the stromal cell layer were also maintained. The cultures were fed 3 times a week with fresh growth medium (α-MEM+ 10% FCS) and were subcultured by using 0.25% trypsin plus 0.02% EDTA in PBS, or by seeding the cells onto other stromal cell layers. These stroma-dependent cells were designated as ELM-D.

Character of ELM-I-1 Cells The doubling time of ELM-I-1 cells is about 24 hr in α-MEM supplemented with 10% FCS.

When these cells were intravenously injected into C3H mice at the dose of 10^4, 10^5 or 10^6 cells per mouse, leukemia was induced in all cases. ELM-D cells also induced leukemia in host mice when they were injected as above.

Epo Receptors (Epo-R) on the Surface of ELM-I-1 Cells Epo promotes the growth of erythroid precursors and supports the differentiation to erythroblasts, in which globin synthesis starts. The action of Epo is known to

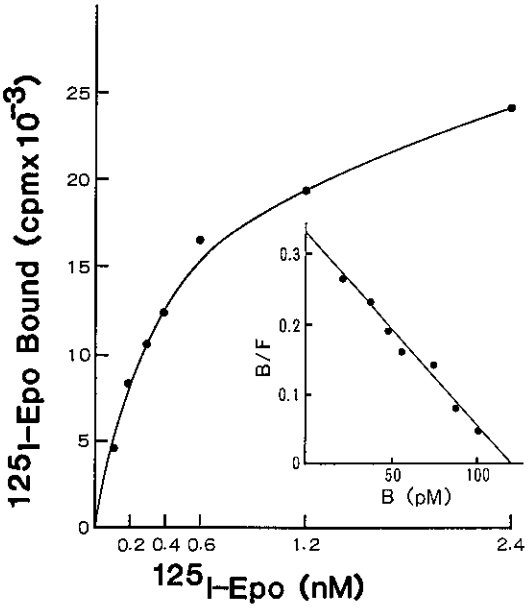

Fig. 2. Concentration-dependent binding of ^{125}I-Epo to ELM-I-1 cells. Specific binding was calculated by subtraction of non-specific binding (in the presence of 200-fold unlabeled Epo) from total binding (in the absence of unlabeled Epo). The inset shows a Scatchard plot [ordinate (B/F), specific binding/free ligand; abscissa (B), specific binding].

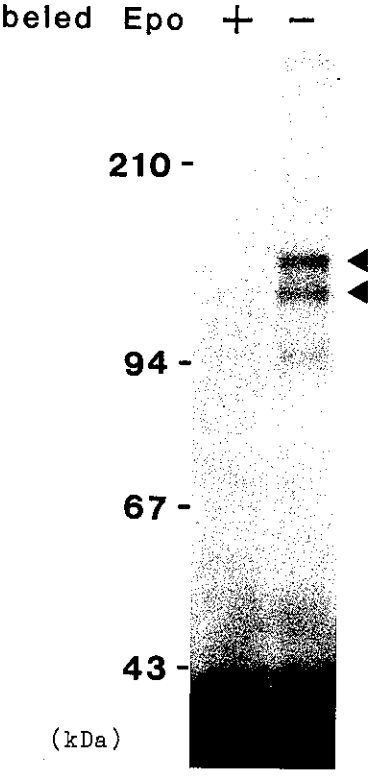

Fig. 3. Analysis of ^{125}I-Epo covalently cross-linked to ELM-I-1 cells by SDS/PAGE under reducing conditions. Procedures for the cross-linking study were as described in "Materials and Methods." For binding of ^{125}I-Epo, the ligand was incubated with ELM-I-1 cells in the presence (+) or absence (−) of 260-fold unlabeled Epo for 1 hr. Arrows (◀) indicate two major radiolabeled products of 120 kDa and 140 kDa.

be mediated by its binding to specific receptors on the surface of erythroid precursor cells.[11, 12] In order to elucidate whether or not ELM-I-1 cells have Epo-R on their surface, binding of Epo to ELM-I-1 cells and cross-linking of ^{125}I-Epo to ELM-I-1 cells were studied.

Figure 2 shows a ligand-saturation curve of ELM-I-1 cells, and Scatchard analysis of the data shows that the number of binding site/cell is 1,730 with a *Kd* of 364p*M*. A cross-linking study revealed two major bands with molecular masses of 120 kDa and 140 kDa under reducing conditions (Fig. 3). These data show that ELM-I-1 cells have Epo-R on their cell surface.

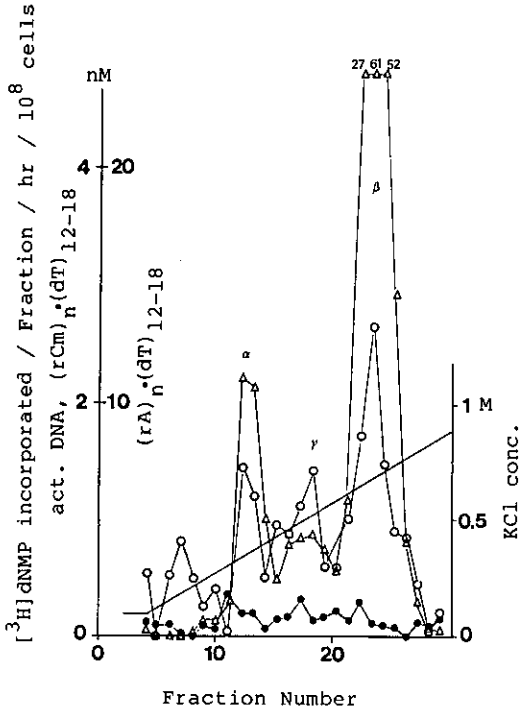

Fig. 4. Phosphocellulose column chromatography of the cell extract from ELM-I-1 cells. Preparation of the cell extract, procedures for chromatography and RT and DNA polymerase assays were carried out as described by Ono *et al.*[2, 9] DNA polymerase activities were measured with activated calf thymus DNA (○), $(rA)_n$·$(dT)_{12-18}$ (△) and $(rCm)_n$·$(dG)_{12-18}$ (●). KCl concentration (—).

Reverse Transcriptase (RT) Activity We have reported previously that this erythroblastic leukemia is clearly distinguishable from those reported by Friend[13] or Rauscher,[14] since no RT activity was detected in the spleen or the liver of the mice.[1]

In the present study, we have also confirmed that RT activity was not detectable in ELM-I-1 cells as well as in the culture media. Figure 4 shows a typical example of phosphocellulose column chromatography of the extract from ELM-1 cells. High activities of cellular DNA polymerases were clearly seen, while RT activity assayed with $(rCm)_n$·$(dG)_{12-18}$ was at the background level throughout all the fractions.

Discussion

Tumor cells are generally regarded as being more adaptable to *in vitro* growth than their normal counterparts, and this has been interpreted as reflecting their capacity to proliferate dominantly *in vivo*. However, only a very small population of leukemic cells actually adapted to *in vitro* growth in many attempts to establish tumor cell lines. Thus, growth of tumoral cells *in vitro* may also depend on the mechanisms which support normal hemopoiesis *in vivo*. Several studies have demonstrated that not only hemopoietic growth factor(s), but also stromal cells play an important role in normal hemopoiesis. Dependence of leukemic cell proliferation on a specific growth factor was demonstrated for T-cell leukemia,[15] acute myeloid leukemia[16–18] and acute lymphoblastic leukemia cells.[19] The importance of stromal cells in culture of lymphoblastic leukemia cells was also reported.[20–23]

Genetically anemic mice of S1/S1^d strain demonstrate the importance of the microenvironment *in vivo*,[24] and adherent stromal cells of murine long-term bone marrow culture are essential in maintaining hemopoiesis *in vitro*.[3, 4] One aim of this study was to clarify the role of stromal cells in the proliferation of leukemic cells.

In the present study, we have demonstrated the importance of stromal cells for *in vitro* growth of the leukemic cells from erythroblastic leukemia. The leukemic cells, termed ELM-D, grow only in close contact with stromal cells (as shown in Table I and Fig. 1),

and the conditioned medium from stromal cell layers did not support the growth of leukemic cells (data not shown). These findings suggest that cell-to-cell interaction between stromal cells and leukemic cells is prerequisite for proliferation of leukemic ELM-D cells, as in normal hemopoiesis.[25] Stroma-independent ELM-I-1 was established from these stroma-dependent cells after 7 weeks of culture on top of marrow stromal cells. Thus, stromal cells appear to be essential for the first few weeks in the culture of erythroid precursor cells. Such a role of the normal hemopoietic microenvironment has previously been demonstrated in Friend virus-induced erythroleukemia by Mager *et al.*[26]

For clear discrimination and identification of the reverse transcriptase (RT) from cellular DNA polymerases, a combination of phosphocellulose column chromatography and species-specific template-primer(s) was shown to be useful in analyzing intracellular RT.[2, 9] Organ distribution of RT in Friend virus-infected mice[27] and RT activity of Rauscher murine leukemia virus-infected culture cells[9] were studied in detail by using this method. Since RT activity was negative in ELM-I-1 cells, which induce leukemia in host mice, it is confirmed that our erythroblastic leukemia is clearly distinct from those reported by Friend or Rauscher.

Epo is known to promote and differentiate erythroid precursor cells. Krantz and Goldwasser[28] and Sasaki *et al.*[8] presented evidence that Epo becomes attached to binding sites on the cell surface. Cross-linking of ^{125}I-Epo to TSA8 cells from Friend leukemia and fetal liver cells has demonstrated the presence of two major radiolabeled products (120 kDa and 140 kDa) on sodium dodecyl sulfate/polyacrylamide electrophoresis (SDS/PAGE) under reducing conditions.[8] In the present study, we also showed that Epo becomes attached to binding sites on the surface of ELM-I-1 cells, and two major species of the cross-linked products with molecular masses of 120 kDa and 140 kDa were detected on SDS/PAGE under reducing conditions. These data are consistent with the previous reports[8] and show that ELM-I-1 cells have Epo-R on their surface.

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