Response of Newly Established Mouse Myeloid Leukemic Cell Lines to MC3T3-G2/PA6 Preadipocytes and Hematopoietic Factors

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Some mouse myeloid leukemias induced by X-irradiation and serially transplanted into syngenic mice do not proliferate in vitro even in the presence of hematopoietic factors. To examine whether such leukemic cells can proliferate in response to stromal cells, we cocultured them with MC3T3-G2/PA6 (PA6) preadipocytes, cells that can support the growth of hematopoietic stem cells. All leukemias developed into in vitro cell lines, showing a dependence on contact with the PA6 cells. Two cell lines responded to none of the known hematopoietic factors including interleukin-3 (IL-3), IL-4, IL-5, IL-6, GM-CSF, G-CSF, M-CSF, and Epo. These results demonstrate that the mechanism of the action of PA6 cells is different from that of any of the known hematopoietic factors, and that, because these two leukemic cell lines retained the ability to grow in vivo, responsiveness to the known hematopoietic factors is not essential for the leukemic cell growth in vivo. Furthermore, all leukemic cell lines could respond also to the preadipocytes fixed with formalin, paraformaldehyde, or glutaraldehyde, suggesting that some molecule(s) associated with the surface of PA6 cells or with extracellular matrix secreted by the preadipocytes is responsible for the leukemic cell growth.

UNDERSTANDING of hematopoiesis has been advanced through the use of two in vitro culture systems, a short-term colony assay in semisolid cultures and a long-term liquid culture system.

In the former system, development of colonies consisting of various combinations of differentiated hematopoietic cells depends on an exogenous supply of humoral hematopoietic factors. Among these factors, interleukin-3 (IL-3) has been demonstrated to act on the progenitors at the earliest stage of hematopoietic cell differentiation.[1,2] However, even this lymphokine cannot support long-term proliferation of hematopoietic stem cells, defined as colony-forming units in spleen (CFU-S).[3-5]

In the latter system, an adherent stromal cell layer is a prerequisite for the maintenance of CFU-S and sustained hematopoiesis over several months.[6] Mouse stromal cell lines that have the capacity to support long-term hematopoiesis have been established.[7-11] Furthermore, cell lines capable of supporting B lymphopoiesis[12-14] or both myeloid and B lymphocyte development under different culture conditions[15-20] have also been isolated from mouse bone marrow cultures.

Our preadipose cell line MC3T3-G2/PA6 (PA6)[21] can support proliferation of CFU-S only when the preadipocytes are in contact with the stem cells,[7] and the entire process of hematopoiesis takes place in intimate association with the preadipocyte layers.[7,22] PA6 cells and 14F1.1 cells[9] do not synthesize a detectable level of messenger RNA for IL-3, IL-4, or granulocyte-macrophage colony-stimulating factor (GM-CSF).[20,23] In addition, Zipori and Lee[20] reported that introduction of the IL-3 gene into 14F1.1 cells does not alter their potential to induce renewal of hematopoietic stem cells in culture. Eliason et al[24] reported that IL-3 is not produced in long-term bone marrow cultures; therefore, IL-3 is unlikely to be involved in the hematopoiesis supported by stromal cells through direct cell-to-cell interaction.

On the other hand, Yoshida et al[25] found that some mouse myeloid leukemias induced by X-irradiation and serially transplanted into syngeneic mice do not proliferate in vitro even when pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM), L-cell conditioned medium or erythropoietin (Epo) is added to the culture.

We speculated that this type of myeloid leukemic cell might proliferate in vivo only in response to stromal cells through cell-to-cell contact. To explore this possibility, we selected four leukemias that show little response to hematopoietic factors and one leukemia responding to PWM-SCM, and cocultured them with PA6 cells. All these leukemias developed into in vitro cell lines, showing a dependence on contact with the preadipocytes. Of these five leukemic cell lines, two responded to none of eight recombinant hematopoietic factors, including IL-3, IL-4, IL-5, IL-6, GM-CSF, granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), and Epo. These findings demonstrate that the mechanism of the action of stromal cells, through direct cell-to-cell interaction, is entirely different from that of any of the known hematopoietic factors.

MATERIALS AND METHODS

Mice. Eight-to-fifteen-week-old female C3H/He mice, either raised in the animal facility of the National Institute of Radiological Science or purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, were used for the transplantation of leukemic cells.

Cell lines. Myeloid leukemias induced in male C3H/He mice by X-irradiation were maintained by serial transplantation into synge-

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neic female mice.[25] PA6 preadipocytes[21] were maintained and subcultured as described previously.[22]

Establishment of leukemic cell lines. All cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Confluent PA6 cell layers were established in 35-mm plastic dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) as reported previously.[22] Leukemic spleens were removed from mice immediately before dying of leukemia. Single cell suspensions of spleen cells were inoculated at $1 \times 10^4 - 1 \times 10^5$ cells/dish onto the preadipocyte layers and cultured with 1.5 mL of α modification of Eagle's minimal essential medium (α-MEM, Flow Laboratories, McLean, VA) supplemented with 10% horse serum (Irvine Scientific, Santa Ana, CA). Cultures were fed twice a week by removal of the medium containing nonadherent cells and addition of fresh medium. After 1 to 2 weeks, the leukemic cells were subcultured by harvesting by vigorous pipetting and seeding onto freshly prepared PA6 cell layers. Then the leukemic cells were repeatedly passaged at 3 to 7 day intervals. Clones were isolated by the limiting dilution method.

Recombinant hematopoietic factors. Purified recombinant human G-CSF (rhG-CSF, 2.5×10^7 U/mg protein), IL-6 (rhIL-6, 3.9×10^9 U/mg protein), and purified recombinant mouse GM-CSF (rmGM-CSF, 3.7×10^8 U/mg protein) were generously provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan), Ajinomoto Pharmaceutical Co Ltd (Kawasaki, Japan), and Sumitomo Pharmaceutical Co Ltd (Osaka, Japan), respectively. Partially purified recombinant human M-CSF (rhM-CSF, 5,400 U/mL) was donated by Dr Yoshikatsu Hirai (Otsuka Pharmaceutical Co Ltd, Tokushima, Japan). Highly purified recombinant human Epo (rhEpo, 81,600 U/mg protein) was a gift of Dr Masatsugu Ueda (Snow Brand Milk Product Co Ltd, Tochigi, Japan). Recombinant mouse IL-3 (rmIL-3), IL-4 (rmIL-4), and recombinant human IL-5 (rhIL-5) were kindly provided by Dr Kem-ichi Arai (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Standard concentrations of hematopoietic factors used in our cultures were as follows: rmIL-3, 100 U/mL; rmIL-4, 0.1%; rhIL-5, 0.5%; rhIL-6, 20 ng/mL; rmGM-CSF, 100 U/mL; rhG-CSF, 20 ng/mL; rhM-CSF, 500 U/mL; and rhEpo, 2 U/mL. We confirmed that the human factors act on normal mouse bone marrow cells.

Cell proliferation assay. Leukemic cells were harvested by vigorous pipetting. After removal of PA6 cell clumps by filtration through mesh, single PA6 cells were removed by 1-hour adherence to a plastic surface. Purified leukemic cells were inoculated at 5×10^4 cells/35-mm dish onto confluent PA6 cell layers or into empty dishes and cultured with 1.5 mL of medium for 3 days in the absence or presence of recombinant hematopoietic factors. Leukemic cells were then harvested by vigorous pipetting, and viable cells were counted in a hemocytometer by means of the dye exclusion test using 0.25% trypan blue solution. Leukemic cells were easily distinguished from the small number of contaminating PA6 cells by their smaller size and smooth and refractile contours.

For the examination of leukemic cell growth under the condition where contact of the leukemic cells with PA6 cells was physically prevented, the preadipocytes were seeded at 3×10^4 cells/slip in 0.5 mL of medium onto Thermanox plastic tissue culture coverslips (26 × 26 mm, Lux Scientific, Newbury Park, CA), previously bent at the four corners and placed in 35-mm dishes. After one day of culture, 1 mL of medium was added. On day 3, some of the coverslips were placed upside down in new 35-mm dishes, and medium was removed from the dishes containing the rest of the coverslips still in their original position. Then 5×10^4 leukemic cells in 1.5 mL of medium were inoculated into all the dishes and cultured. In dishes where coverslips had been placed upright, leukemic cells could make contact with the PA6 cells, whereas in dishes containing coverslips that had been inverted, they could not. As controls, the same number of leukemic cells alone was cultured. After 3 days of culture, the leukemic cells were harvested and counted as described above.

Fixation of PA6 cells. Confluent PA6 cell layers established in 35-mm dishes were washed 3 times with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS^-). They were then fixed with either 5% formalin, 5% paraformaldehyde, or 0.05% glutaraldehyde in PBS^- at room temperature for 20 minutes. After being washed 3 times with PBS^- and once with medium, these fixed preadipocytes were used for the coculture experiments with leukemic cells.

Assay of CSF. Medium was conditioned for 3 days (CM) with either untreated or fixed confluent PA6 cell layers. CSF was assayed in a methylcellulose culture system by culturing of 5×10^4 bone marrow cells with 20% CM as described previously.[7]

RESULTS

Establishment of leukemic cell lines. Based on preliminary examinations of transplantable mouse myeloid leukemias using various conditioned media, we selected four leukemias, including L-8002 (erythroblastic), L-8027 (myelomonocytic), L-8065 (monocytic), and L-8733 (myelomonocytic), showing little response to hematopoietic factors, and one leukemia, L-8010 (myelomonocytic) that responded to PWM-SCM, and cocultured them with PA6 cells. Cells of all leukemias actively proliferated within the cocultures and developed into in vitro cell lines, designated L-8002c, L-8010c, L-8027c, L-8065c, and L-8733c. Subsequently we isolated clones, L-8002c-2, L-8010c-3, L-8027c-3 and L-8733c-23, by the limiting dilution method. Cells of these leukemic cell lines retained morphologic and cytochemical characteristics of their original myeloid leukemias (data not shown).

When 5×10^6 cells of the leukemic cell lines were intravenously injected into syngeneic mice (five mice for each leukemic cell line), all but those into which L-8733c-23 cells had been injected died of leukemia within 3 weeks (data not shown), indicating that our leukemic cell lines, other than the L-8733c-23 cell line, still retained their ability to proliferate in vivo and kill their hosts. The reason L-8733c-23 cells do not proliferate in vivo is not clear.

Dependence of survival and proliferation of the leukemic cells on contact with PA6 cells. Representative growth curves of our leukemic cell lines in the absence or presence of PA6 cells are shown in Figs 1A and 1B. Leukemic cells proliferated exponentially within the cocultures with nontreated PA6 cells. In contrast, when the leukemic cells were cultured alone, the number of cells gradually decreased.

Although our earlier work showed that, within the cocultures of bone marrow cells and PA6 cells, most normal immature hematopoietic cells were distributed between layers of the multi-layered preadipocytes,[22] leukemic cells tended to attach to the superficial layer only. Therefore, the nature of the interaction of the leukemic cells with PA6 cells appears to be somewhat different from that of normal hematopoietic progenitors. Nevertheless, cells of all of leukemic cell lines actively proliferated only when the leukemic cells were allowed to make physical contact with the preadipocytes (Table 1). Although the cell number of some of the leukemic cell lines increased nearly twofold despite the absence of PA6 cells, even in such cases the cells

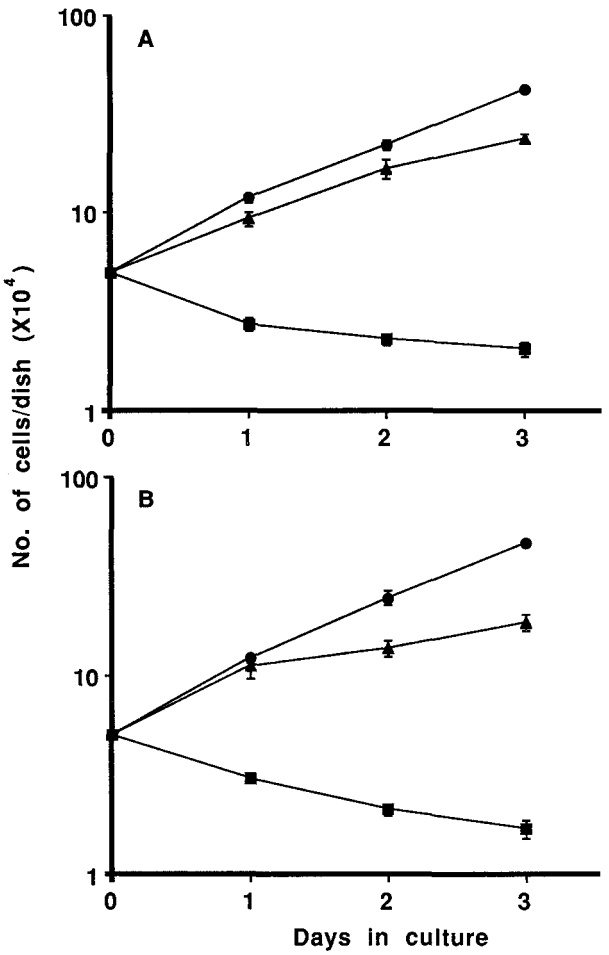

Fig 1. Growth curves of leukemic cells cultured in the absence or presence of untreated or formalin-fixed PA6 cells. L-8027c-3 (A) or L-8065c (B) leukemic cells were inoculated into empty dishes (■) or onto untreated (●) or formalin-fixed (▲) PA6 cells and cultured. Each point shows the mean ± SD of counts from three dishes. Differences in leukemic cell numbers after 3 days of culture under the three different culture conditions were significant ($P < .001$, by Student's t test).

died off within 1 week (data not shown). These results show that none of our leukemic cell lines has yet acquired the ability to proliferate autonomously, and that direct cellular interaction with PA6 cells is essential for the survival and proliferation of the leukemic cells.

Responsiveness of the leukemic cell lines to hematopoietic factors. We examined the responsiveness of our leukemic cell lines to known hematopoietic factors including IL-3, IL-4, IL-5, IL-6, GM-CSF, G-CSF, M-CSF, and Epo. As shown in Table 2, L-8027c-3 cells and L-8065c cells responded to none of the eight recombinant factors at preexamined concentrations optimal for the corresponding normal target hematopoietic cells. Furthermore, these leukemic cells did not respond to 10-fold higher and lower concentrations of rmIL-3, rmGM-CSF, and rhG-CSF (data not shown). These results eliminate the possibility that the preadipocytes support leukemic cell growth by producing some of the known hematopoietic factors, and are consistent with the above result that PA6 cells can support leukemic cell growth only through direct cell-to-cell contact (Table 1).

The three other leukemic cell lines responded to both PA6 cells through direct cellular interaction and to one or more of the known hematopoietic factors (Table 2). L-8002c-2 cells showed a response only to rhEpo, and it was quite low. L-8010c-3 cells, derived from the leukemia showing a response to PWM-SCM, responded to rmGM-CSF, rhG-CSF, and rmIL-3, and weakly to rmIL-4. In addition, L-8733c-23 cells unexpectedly responded to rmGM-CSF to the same extent as to PA6 cells through cell-to-cell contact, and to rhG-CSF to a lesser extent. Consistent with our previous findings that the preadipocytes do not constitutively synthesize a detectable level of messenger RNA for IL-3, IL-4 and GM-CSF[23] are the results showing that leukemic cells responding to IL-3, IL-4, GM-CSF, and G-CSF responded to PA6 cells only through cell-to-cell contact (Tables 1 and 2).

Effect of fixation of PA6 cells on their ability to support the proliferation of the leukemic cells. Although results shown in Table 1 demonstrate that PA6 cells do not constitutively produce any humoral factor that supports growth of the leukemic cells we examined, through direct cell-to-cell interaction, the leukemic cells may induce production of some factor(s) by the preadipocytes and respond to it, when the leukemic cells are cocultured with PA6 cells. Therefore, we examined whether PA6 cells metabolically inactivated by fixation with formalin or other fixatives could support leukemic cell growth. When leukemic cells were cocultured with PA6 cells fixed with 5% formalin, they adhered to the fixed preadipocyte layers and proliferated, although they were easily detached from the preadipocyte layers by

Table 1. Requirement of Cell-to-Cell Contact With PA6 Cells for Leukemic Cell Growth

Cell Line	PA6 Cells	Contact	No. of Cells/Dish (× 10⁴)
L-8002c-2	−		11 ± 3.6
	+	−	8.2 ± 2.3
	+	+	58 ± 3.3
L-8010c-3	−		13 ± 1.7
	+	−	18 ± 1.0
	+	+	77 ± 0.4
L-8027c-3	−		2.1 ± 0.1
	+	−	6.3 ± 1.1
	+	+	60 ± 2.8
L-8065c	−		2.1 ± 0.1
	+	−	2.1 ± 0.2
	+	+	26 ± 1.3
L-8733c-23	−		4.2 ± 0.2
	+	−	8.5 ± 0.5
	+	+	45 ± 4.0

Values are means ± SD of counts from three dishes. Numbers of leukemic cells harvested after cells had been cultured in contact with PA6 cells were significantly different from those of cells harvested after having been cultured under the other two culture conditons ($P < .001$, by Student's t test). Differences between the leukemic cell numbers after the cells had been cultured alone and those after having been cultured without contact with PA6 cells were less significant ($P > .01$).

Table 2. Response of Leukemic Cell Lines to Various Recombinant Hematopoietic Factors

Addition	No. of Cells/Dish (× 10^4)				
	L-8002c-2 Cells	L-8010c-3 Cells	L-8027c-3 Cells	L-8065c Cells	L-8733c-23 Cells
None	11 ± 0.1	12 ± 0.1	9.0 ± 0.2	2.1 ± 0.1	4.1 ± 0.2
PA6 cells	60 ± 4.4	78 ± 1.0	43.0 ± 3.0	21.0 ± 0.3	43.0 ± 1.0
rmIL-3	11 ± 0.3	26 ± 1.5	9.6 ± 0.6	2.3 ± 0.1	6.4 ± 0.2
rmIL-4	11 ± 0.7	18 ± 1.0	9.3 ± 0.8	2.4 ± 0.2	4.2 ± 0.2
rmIL-5	11 ± 0.8	12 ± 1.3	9.5 ± 0.5	2.4 ± 0.2	4.0 ± 0.3
rhIL-6	11 ± 0.5	13 ± 0.7	9.7 ± 0.6	2.2 ± 0.2	3.4 ± 0.6
rmGM-CSF	11 ± 0.7	69 ± 6.7	11.0 ± 0.5	2.2 ± 0.2	41.0 ± 3.5
rhG-CSF	12 ± 0.3	28 ± 2.6	9.5 ± 1.2	2.3 ± 0.1	22.0 ± 1.6
rhM-CSF	12 ± 0.2	13 ± 1.6	9.1 ± 0.9	2.1 ± 0.1	4.1 ± 0.3
rhEpo	18 ± 1.9	12 ± 1.1	11.0 ± 0.9	2.1 ± 0.3	3.1 ± 0.3

Values are means ± SD of counts from three dishes.

pipetting and responded to the fixed PA6 cells to a somewhat lesser degree than to the untreated preadipocytes (Figs 1A and B). PA6 cells fixed with 5% paraformaldehyde or 0.05% glutaraldehyde also retained their ability to support leukemic cell growth (Table 3). No fixed PA6 cells excluded trypan blue. CSF activity was not detected in the conditioned media of these fixed PA6 cells (Table 4). No hematopoietic cell growth was observed within the cocultures of normal bone marrow cells and formalin-fixed PA6 cells (data not shown). These results demonstrate that the fixation procedure effectively kills PA6 cells but does not abrogate their capacity to interact with leukemic cells and support leukemic cell growth, and that proliferation of leukemic cells does not depend on their ability to induce the production of some factor by PA6 cells.

DISCUSSION

In the present study, we found that all five transplantable mouse myeloid leukemias we examined retained responsiveness to PA6 preadipocytes. In vitro cell lines were developed from all such leukemias by coculturing with the preadipocytes. Cells of all the cell lines showed a dependence on PA6 cells and responded to PA6 cells exclusively through direct cell-to-cell interaction. In our previous studies, we could not completely eliminate the possibility that some of the activity of PA6 cells to support the proliferation of CFU-S and their differentiation may have been due to some cells having been derived from bone marrow, because we had not used a purified CFU-S population for the coculture experiments with PA6 cells.[7,22] However, our present results showing that clonal leukemic cell lines can respond to PA6 cells demonstrate that PA6 cells alone can support leukemic cell growth without any involvement of other types of cells.

Two of our leukemic cell lines, L-8027c-3 and L-8065c, had lost their responsiveness to all of the humoral factors previously identified to directly affect hematopoietic progenitors at various stages of differentiation, including IL-3, IL-4, IL-5, IL-6, GM-CSF, G-CSF, M-CSF, and Epo. Although some stromal cell lines derived from bone marrow have been shown to secrete interleukin-7 (IL-7), a factor that is requisite for B lymphopoiesis,[26-28] PA6 cells produce IL-7 neither constitutively nor in response to inducers.[28] Thus it is hardly conceivable that the function of PA6 cells to support proliferation of these leukemic cells through direct cellular interaction is mediated either by production of some of the known hematopoietic factors or by a mechanism similar to that of the factors.

The responsiveness to the known hematopoietic factors is not always a prerequisite for the in vivo proliferation of myeloid leukemic cells, as shown by our finding that the leukemic cell lines not responding to any of the known hematopoietic factors retained their ability to proliferate in vivo and kill their hosts. In contrast, committed IL-3-dependent cell lines, such as DA-1, FDC-P2, and 32D C13, have lost their ability to respond to stromal cells,[23,29] and FDC-P2 cells cannot proliferate in vivo.[30] Therefore, the ability to respond to stromal cells through cell-to-cell contact, rather than the responsiveness to humoral hematopoietic factors, may be crucial for the in vivo proliferation of myeloid leukemic cells before they acquire the ability to proliferate autonomously, although our present survey of transplantable mouse leukemias is not enough.

Table 3. Effect of Fixation of PA6 Cells on Their Ability to Support the Leukemic Cell Growth

PA6 Cells	Fixation	No. of Cells/Dish (× 10^4)		
		L-8010c-3 Cells	L-8065c Cells	L-8733c-23 Cells
−	—	9.8 ± 0.4	3.7 ± 0.3	3.6 ± 0.4
+	—	62 ± 4.4	25 ± 1.6	44 ± 5.0
+	Formalin	52 ± 3.2	19 ± 1.1	38 ± 1.4
+	Paraformaldehyde	54 ± 3.2	19 ± 1.5	36 ± 1.6
+	Glutaraldehyde	52 ± 4.0	16 ± 2.6	35 ± 1.9

Values are means ± SD of counts from three dishes.

Table 4. Effect of Fixation of PA6 Cells on Their Ability to Secrete CSF

CM	No. of Colonies/Dish
None	0
Untreated PA6 cells	61 ± 4.9
Formalin-fixed PA6 cells	1 ± 0.8
Paraformaldehyde-fixed PA6 cells	1 ± 0.8
Glutaraldehyde-fixed PA6 cells	0.7 ± 0.6

Values are means ± SD of counts from three dishes. Difference in the number of colonies formed in the absence or presence of CM of fixed PA6 cells was not significant ($P > .05$, by Student's t test).

Although our previous[7,23] and present data demonstrated that PA6 cells do not constitutively secrete factors that support the proliferation of CFU-S and our leukemic cells, these results do not rule out the possibility that CFU-S and the leukemic cells may stimulate PA6 cells, through direct cell-to-cell contact, to produce some factor(s) and respond to it. Indeed, IL-7-responsive B-cell lines are shown to be potent inducers of IL-7 production by the ST2 stromal cell line.[28] Roberts et al[29] reported that IL-3-dependent multipotential stem cell lines (FDC-Mix) respond to both untreated and glutaraldehyde-fixed Swiss/3T3 cells through cell-to-cell contact. Therefore, we examined whether metabolically inactive PA6 cells could support the leukemic cell growth. Fixation of PA6 cells with 5% formalin, 5% paraformaldehyde or 0.05% glutaraldehyde only slightly diminished their ability to interact with leukemic cells and support the leukemic cell proliferation, whereas the fixation procedure was adequate to stop the secretion of CSF by PA6 cells. These findings further demonstrate that PA6 cells can support the proliferation of our leukemic cells without any involvement of humoral factors produced by the preadipocytes. We suggest that some specific molecule(s) associated with either the surface membrane of PA6 cells or extracellular matrix produced by the preadipocytes is responsible for the ability of PA6 cells to support the leukemic cell growth. If this is true, the present results show that the molecule(s) is active even when immobilized and can give a signal(s) to leukemic cells to proliferate.

Our present study has strongly suggested that not only PA6 cell line and other stromal cell lines but also our leukemic cell lines provide powerful tools for elucidation of the molecular mechanism of cell-to-cell interaction between hematopoietic cells and stromal cells within hematopoietic organs, although it is not yet certain whether PA6 cells affect normal and neoplastic hematopoietic cells through exactly the same mechanism. Our efforts to verify the hypotheses offered above and to identify the molecules responsible for the function of stromal cells to support hematopoietic cell proliferation are now in progress.

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