[CANCER RESEARCH 47, 2879-2882, June 1, 1987]

Effect of Direct Cell-to-Cell Interaction between the KM-102 Clonal Human Marrow Stromal Cell Line and the HL-60 Myeloid Leukemic Cell Line on the Differentiation and Proliferation of the HL-60 Line[1]

Hiroyuki Ohkawa and Kenichi Harigaya[2]

Department of Pathology, Keio University School of Medicine, Shinjiku-ku, Tokyo 160, Japan

ABSTRACT

Hematopoietic cellular interaction was investigated in a coculture of the human clonal marrow stromal line, KM-102, and the myeloid leukemia cell line, HL-60. In the coculture, a large number of HL-60 cells remained in the supernatant but some of them became firmly attached to KM-102 cells. The attached HL-60 cells showed little positive reaction in the NBT test when the culture was supplemented with 10^{-9} to 10^{-7} M $1\alpha,25$-dihydroxyvitamin D_3. In contrast, differentiation in the supernatant HL-60 cells was strikingly responsive to the agent in a dose-dependent way. Furthermore, the complete inhibition was observed in the incorporation of [^{3}H]thymidine into the attached HL-60 cells with autoradiography, but 23.6% of the supernatant cells moderately incorporated [^{3}H] thymidine into their nuclei. There was no attachment between HL-60 cells and stromal cells from human thymus and lymph node, or between lymphocytic leukemia cells and marrow stromal cells. These results indicate that there is direct cellular interaction between myeloid leukemic cells and marrow stromal cells which modulates the proliferation and differentiation of the myeloid leukemic cells. This modulation by marrow stromal cells is more strongly affected by this interaction than by exogenously added differentiation-inducing agents. Apparently marrow stromal cells produce a definitive milieu for the proliferation and differentiation of myeloid leukemic cells.

INTRODUCTION

It has been suggested that hematopoiesis is controlled by hematopoietic diffusible factors (1) and cell-to-cell interaction (2) between hematopoietic cells and marrow stromal cells. The development of a long-term culture system using bone marrow liquid (3, 4) has shed light on the hematopoietic and stromal interaction. However, since the culture is composed of different types of cells, the precise nature of the cell-to-cell interaction has not been clear. It has been reported that the inhibition of granulocytic/macrophage (G/M) colony formation requires close contact between the adherent bone marrow cells and the granulocyte-macrophage stem cells (CFUc) in both human and murine cultures, since no inhibition occurred when a thin agar layer separated the adherent cells from the target bone marrow cells (5, 6). Therefore, the establishment of clonal stromal cell lines (7–9) makes it possible to explore the hematopoietic stromal functions using simple combinations of effector cells and target cells.

The present experiment was carried out to investigate the cell-to-cell interaction in the hematopoietic microenvironment *in vitro* using a coculture system with clonal human marrow stromal cell line and leukemic myeloid cells. The results show clearly that the proliferation and differentiation of HL-60 cells are inhibited when they are attached to marrow stromal KM-102 cells.

MATERIALS AND METHODS

Culture of Cells. The HL-60 (10) was maintained in Iscove's modification of Dulbeco's medium with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin at 37°C, 7.5% CO_2, and 100% humidity. The cells were transferred into new flasks at a density of 2.0×10^5 cells/ml every 3 days. The KM-102 cells, one of the human clonal stromal cell lines (7), were established by transfection of a viral oncogene. They were cultured in the same way as the HL-60 cells, and subcultured at a split ratio of 1:8 every 5 days using trypsin EDTA dispersant, replaced with fresh medium every 3 days.

Supplements. $1\alpha,25(OH)_2D_3$[3] was the gift of Mr. Fukushima (Chugai Pharmaceutical Co. Ltd., Tokyo). $1\alpha,25(OH)_2D_3$ was finally diluted at 10^{-9} to 10^{-7} M in the growth medium on every supplementation. The medium conditioned by KM-102 cells was collected on the 4th day after confluence. The conditioned medium was centrifuged at $800 \times g$ for 10 min, filtered through a 0.2 μm membrane filter (Millipore, Bedford, MA, and stored at −20°C.

Effect of KM-102 Cells on the Differentiation of HL-60 Cells by $1\alpha,25(OH)_2D_3$. KM-102 cells were inoculated over sterile cover slips (diameter, 10 mm) in cell wells (24-well; Corning, Corning, NY) at 2.0×10^5 cells/well (Fig. 1). On the third day the cultures were replaced with fresh medium, then HL-60 cells were overlaid on the KM-102 cells at a concentration of 2.0×10^5 cells/ml. After 24 h the supernatant HL-60 cells were harvested and the cover slips were gently agitated and washed twice. Then the cover slips were transferred into new cell wells and fed with fresh growth medium. The supernatant cells were centrifuged at $800 \times g$ for 10 min and inoculated at 2.0×10^5 cells/ml in milli cells (Nihon Millipore Kogyo K. K. Yonezawa) which were set into the wells of the new cell wells. These cultures were supplemented with 10^{-9}, 10^{-8}, or 10^{-7} M $1\alpha,25(OH)_2D_3$. The same volume of the growth medium without $1\alpha,25(OH)_2D_3$ was added to the control culture. Diffusible substances permeated the membrane filter between the two compartments at the bottom of the milli cells, but cells did not. After an additional 3 days of cultures the supernatant HL-60 cells and the cells attached to the KM-102 cells and to the membranes of the milli cells were treated with Nitroblue tetrazolium solution (Sigma Chemical Company, St. Louis, MO) (11).

Effect of KM-102 Cells on the Proliferation of HL-60 Cells. Incorporation of [$_3$H]thymidine into the nuclei of HL-60 cells was studied by autoradiography. [^{3}H]Thymidine (0.8 μCi/ml; specific activity, 78.0 Ci/mM; New England Nuclear, Boston, MA) in 2-ml aliquots was added to each well on the 7th day (Fig. 1) and the wells were incubated for 60 min at 37°C. The supernatant HL-60 cells in the milli cells were centrifuged at $800 \times g$ for 10 min and prepared as smears. Then the cover slips in this culture were washed with plain Iscove's medium without thymidine. The smears and cover slips were dried in air and stored dry at 4°C. All smears and cover slips were dipped in MNR 40 nuclear track emulsion (Konisiroku Photo Ind. Co. Ltd., Tokyo, Japan), diluted with distilled water (1:1), and placed in a 44°C water bath. Thereafter, they were exposed at room temperature for 48 h, developed, and counter stained through the emulsion. At least 200 HL-60 cells were scored for each labeling index value. In the cover slip specimens, 200 labeled and unlabeled HL-60 cells directly in contact with KM-102 cells were counted. Furthermore, to determine whether the HL-60 cells

Received 6/17/86; revised 2/4/87; accepted 2/25/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants-in-Aid for Scientific Research and Cancer Research 59480153, 59015090, and 61015094 from the Ministry of Education, Science, and Culture of Japan.

[2] To whom requests for reprints should be addressed, at the Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjiku-ku, Tokyo 160, Japan.

[3] The abbreviation used is: $1\alpha,25(OH)_2D_3$, $1\alpha,25$-Dihydroxyvitamin D_3.

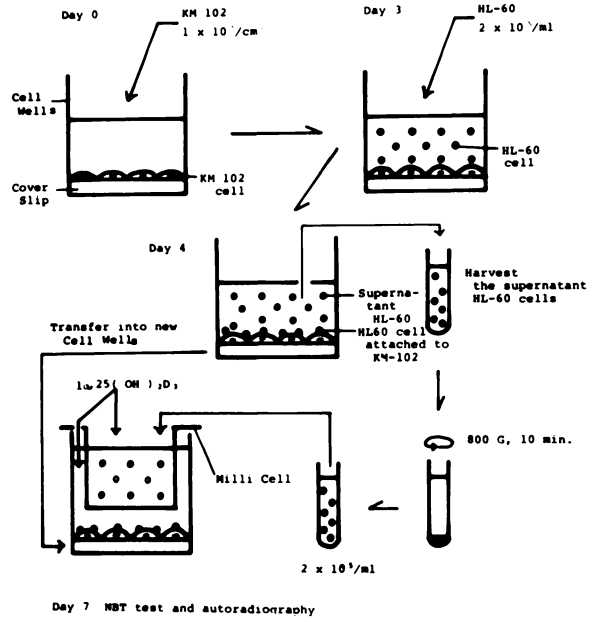


Fig. 1. Outline of experimental procedure.

attached to the cover slips were able to incorporate [^{3}H]thymidine when they were treated with 1α,25(OH)$_2$D$_3$ for 3 days, they were so treated and then autoradiographed to determine the treatment effect.

Dye Transfer. The dye transfer test (12) using Lucifer Yellow CH (Sigma) was used to determine the existence of direct communication mediated by cell junctions between the KM-102 and HL-60 cells.

Various Combinations of Attachments in the Cocultures. Similar coculture experiments were performed using combinations of human thymic (R-3-2) and lymphoid stromal cell lines (SG) with HL-60 cells, and using KM-102 cells with cloned T- (13) and B- (14) cell lines (Table 3). To determine whether the supernatant HL-60 cells and HL-60 cells detached from KM-102 by light trypsinization could attach to KM-102 cells when they were secondarily cocultured, the following experiments were performed. The HL-60 cells attached to KM-102 were harvested from the coculture on the 3rd day by light trypsinization, and it was determined whether with 1α,25(OH)$_2$D$_3$ treatment they reattached to KM-102 and differentiated into more mature cells.

RESULTS

Effect of KM-102 Cells on Differentiation of HL-60 Cells Treated with 1α,25(OH)$_2$D$_3$. According to the NBT reduction test, the positive reaction of supernatant HL-60 cells increased in the milli cells with increased concentrations of 1α,25(OH)$_2$D$_3$. Positive reactions of 18.3, 40.1, and 60.4%, respectively, occurred in the cultures supplemented with 10^{-9}, 10^{-8}, and 10^{-7} M 1α,25(OH)$_2$D$_3$ (Table 1, Fig. 2*a*). The differences among these values were statistically significant ($P < 0.01$) and they also differed from the control cultures. The supernatant HL-60 cells reacted more positively in the culture with feeder cells than in the one without feeder cells ($P < 0.01$). But no reaction products were found in HL-60 cells attached to the KM-102 cells on the cover slips at the given doses of 1α,25(OH)$_2$D$_3$ (Fig. 2*b*). HL-60 cells adhering to the membrane filter of the milli cells showed positive reactions (Fig. 3) dose dependently, as in the supernatant HL-60 cells in the milli cells. The HL-60 cells detached from KM-102 by light trypsinization were able to differentiate into NBT-positive cells when they are treated with 1α,25(OH)$_2$D$_3$.

The medium conditioned by KM-102 cells did not inhibit the differentiation of HL-60 cells in concentrations of 1α,25(OH)$_2$D$_3$ ranging from 2.5 to 30% (data not shown).

Table 1 *Effect of marrow stromal cell line (KM-102) on the 1α,25(OH)$_2$D$_3$-induced differentiation of HL-60 cells*

KM-102 cells	Concentration of D$_3$[a] (M)	NBT reduction (%) Supernatant HL-60 cells	NBT reduction (%) HL-60 cells attached to KM-102
1. −	0	3.1 ± 0.4[b]	
2. −	10^{-8}	26.4 ± 4.1[c]	
3. +	0	3.3 ± 0.8	0
4. +	10^{-9}	18.3 ± 1.8[c,d]	0
5. +	10^{-8}	40.1 ± 3.0[c,d]	0
6. +	10^{-7}	60.4 ± 0.7[c,d]	0

[a] D$_3$, 1α25(OH)$_2$D$_3$.
[b] Values are expressed as mean ± SD of the pooled supernatant HL-60 cells of four experiments.
[c] Statistically significant difference ($P < 0.01$) *vs.* nonsupplemented cultures without KM-102 cells (No. 1).
[d] Significant differences ($P < 0.01$) among supplemented cultures and supplemented cultures *vs.* nonsupplemented cultures with KM-102 cells (No. 3).

Effect of KM-102 Cells on Proliferation of HL-60 Cells. In the cultures supplemented with 1α,25(OH)$_2$D$_3$, the proliferation of supernatant HL-60 cells was inhibited dose dependently. In the cocultures, 23.6% of the supernatant HL-60 cells were labeled with [^{3}H]thymidine (Table 2), but the cells attached to KM-102 cells had no radioactive thymidine. The incorporation of [^{3}H]thymidine into supernatant HL-60 cells was significantly lower in the cultures with KM-102 cells than in the cultures without the cell line feeder. The HL-60 cells attached to cover slips incorporated [^{3}H]thymidine when their cultures contained 1α,25(OH)$_2$D$_3$.

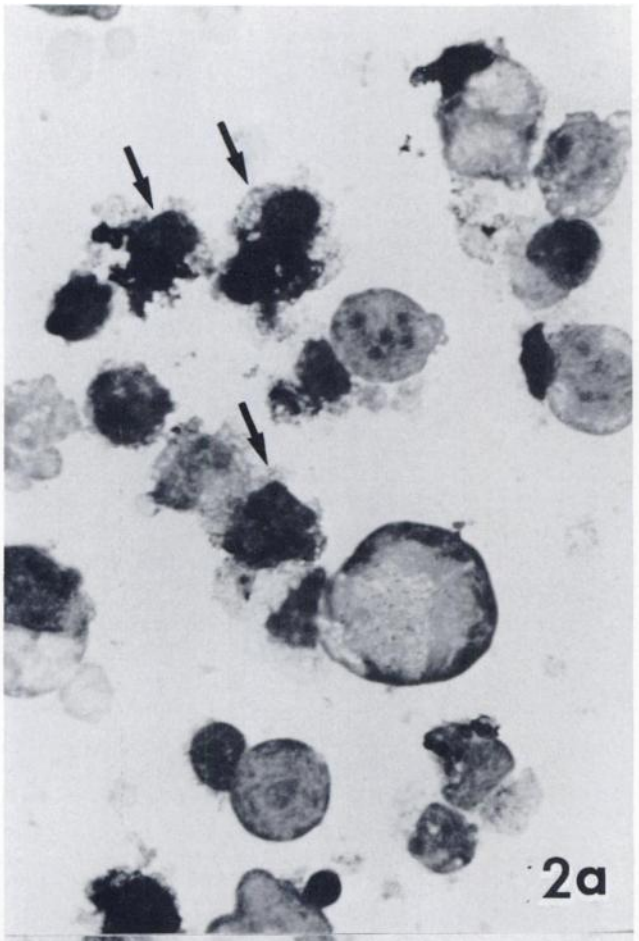
Dye Transfer Test. Dye transfer into adjacent cells was frequently observed in primary bone marrow stromal cells and clonal marrow stromal cells. Sometimes the dye injected into stromal cells moved into attached round hematopoietic cells in primary bone marrow culture. The dye transfer also occurred between KM-102 cells and HL-60 cells, but the frequency was quite low.

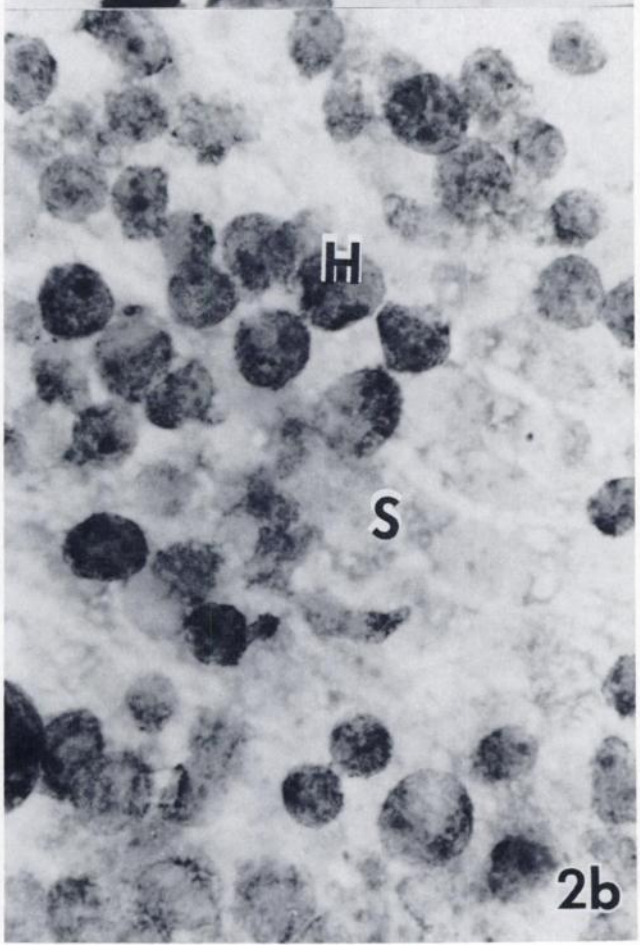
Various Combinations of Stromal Cells and Hematopoietic Cell Lines in the Cocultures. Cells of neither lymphoid cell line adhered to KM-102 cells, nor did stromal cells derived from lymph node and thymus become attached to HL-60 cells. Only the particular combination of KM-102 and HL-60 cells resulted in attachments (Table 3). Moreover, HL-60 cells transferred two or three times were capable of reattachment to the freshly prepared KM-102 feeders. And the HL-60 cells detached from KM-102 by light trypsinization are capable of attachment to freshly prepared feeder cells.

DISCUSSION

Hematopoietic cellular interaction was investigated in a coculture of the human clonal marrow stromal line, KM-102, and the myeloid leukemic cell line, HL-60. A large number of HL-60 cells remained in the supernatant but some of them became firmly attached to KM-102 cells. The stable attachment was observed for 2 weeks. Also, some of the supernatant HL-60 cells transferred two or three times and the cells detached from KM-102 by light trypsinization are capable of attachment to freshly prepared KM-102. Leukemic-T and B-cell lines did not become attached to KM-102 cells in similar coculture systems, nor did HL-60 cells become attached to stromal cells derived from either human lymph node or thymus. Therefore, this attachment appears to be specific for myeloid leukemic cells and marrow stromal cells.

HL-60 is one of a comparatively small number of human leukemic cell lines that are suitable for studying the chemically





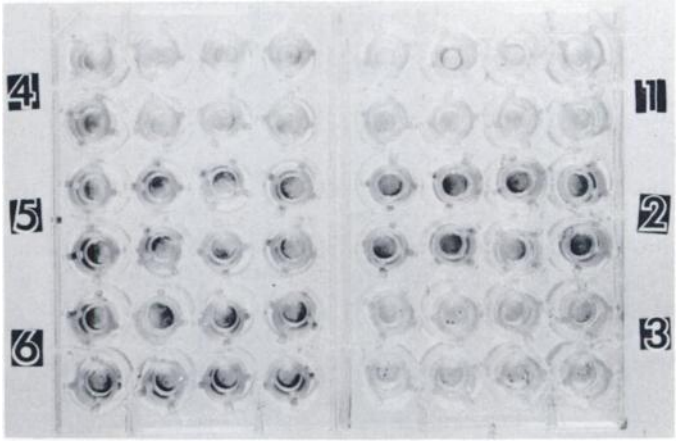


Fig. 3. NBT reduction test in HL-60 cells adhering to the milli cell membrane filters. The concentrations of supplemented $1\alpha,25(OH)_2D_3$ in the milli cell are: *1* and *3*, growth medium only; *4*, 10^{-9} M; *2* and *5*, 10^{-8} M; *6*, 10^{-7} M.

Table 2 *Effect of marrow stromal cell line (KM 102) on the proliferation of HL-60 cells*

KM 102 cells	Concentration of D_3[a] (M)	Percentage of labeling: Supernatant HL-60 cells	Percentage of labeling: HL-60 cells attached to KM-102
1. –	0	47.1 ± 3.0[b]	
2. –	10^{-8}	18.3 ± 2.4[c]	
3. +	0	23.6 ± 1.6[c]	0
4. +	10^{-9}	20.3 ± 3.4[c]	0
5. +	10^{-8}	17.6 ± 2.4[c,d]	0
6. +	10^{-7}	5.2 ± 1.1[c,d]	0

[a] D_3, $1\alpha,25(OH)_2D_3$.
[b] Mean ± SD for eight cultures of rates of labeled HL-60 cells.
[c] Significant difference ($P < 0.01$) *vs.* nonsupplemented coculture without KM-102 cells (No. 1).
[d] Significant difference ($P < 0.01$) *vs.* nonsupplemented coculture with KM-102 cells (No. 3).

Table 3 *Result of attachment in coculture in different combinations of human stromal cell lines with HL-60, and KM-102 cells with leukemia cell lines*

Stromal cell lines	Supernatant cell lines[a]	Attachment
KM-102	HL-60	Yes
KM-102	TALL	No
KM-102	BALL	No
R-3-2 (thymic)	HL-60	No
SG (lymph node)	HL-60	No

[a] TALL, RPMI 8402, human T-cell acute lymphoblastic leukemia; BALL, B-1, human B-cell acute lymphoblastic leukemia.

induced differentiation of myeloid cells *in vitro* (15, 16). HL-60 cells contain cytoplasmic receptors for $1\alpha,25$-dihydroxyvitamin D_3 (17) and they differentiate into more mature monocytic cells when exposed to this vitamin (18). The vitamin D_3-supplemented coculture was carried out in order to examine the cellular interaction between myeloid leukemic cells and marrow stromal cells. This study clearly showed that proliferation and differentiation of HL-60 cells were inhibited when they became attached to marrow stromal KM-102 cells, whereas supernatant HL-60 cells proliferated and differentiated into more mature monocytic cells. Attachment of HL-60 to the surface of KM-102 cells is sufficient to prevent incorporation of [^{3}H]thymidine, but attachment of HL-60 to cover slips does not prevent incorporation. The HL-60 cells detached from KM-102 by light trypsinization are able to differentiate into NBT-

Fig. 2. NBT reduction test in the coculture of HL-60 and KM-102 containing 10^{-8} M $1\alpha,25(OH)_2D_3$, *A*, HL-60 cells in the supernatant, black reaction products are demonstrated in the cytoplasm of HL-60 cells (*arrows*). *B*, HL-60 cells attach to KM-102 cells: *H*, HL-60 cells; *S*, stromal cells. No reaction products are found in HL-60 cells. Giemsa counterstain. × 900.

positive cells when they are treated with $1\alpha,25(OH)_2D_3$. Accordingly, the clonal human stromal cell line inhibited the proliferation and differentiation of the leukemic myeloid cell line by direct contact.

The incorporation of [^{3}H]thymidine into the supernatant HL-60 cells was significantly lower in the culture with KM-102 cells than in the cultures without the cell line. Apparently KM-102 produced a labile substance that inhibited proliferation, although the medium conditioned by these cells did not have any effect on the proliferation and differentiation of HL-60 cells (data not shown).

The number of supernatant HL-60 cells that reacted with the NBT solution increased in the milli cells more than the HL-60 cells without the cell line feeder. This indicates that KM-102 cells yield diffusible factors which stimulate the $1\alpha,25(OH)_2D_3$-induced differentiation of supernatant HL-60 cells.

Dye transfers were observed between KM-102 and HL-60 cells, and this indicates that there is a direct communication of low molecular substances between them. But in view of low frequency, this communication may not be a major inhibitory mechanism in this coculture system. This study indicates that direct interaction occurs between HL-60 cells and marrow stromal cells through their attachment and this is a specific functional phenomenon possibly attributable to extracellular matrix and surface antigens of stromal cells in the hematopoietic microenvironment. This study also revealed that the hematopoietic regulation of neoplastic myeloid cells by marrow stromal cells was stronger than that of the exogenously added differentiation-inducing agent as has been described in normal marrow cells (5, 6). Lately, differentiation-inducing substances have been used for therapeutic applications in leukemic patients (19). However, it should be noted that the proliferation and differentiation of leukemic cells *in vivo* are strongly modulated by a population of marrow stromal cells.

REFERENCES

1. Bradley, T. R., and Metcalf, D. The growth of mouse bone marrow cells *in vitro*. Aust. J. Exp. Biol. Med. Sci., *44:* 287–300, 1966.
2. Schofield, R. The relationship between the spleen colony forming cell and hematopoietic stem cell. A hypothesis. Blood Cells, *4*: 7–25, 1978.
3. Dexter, T. M., and Moore, M. A. S. *In Vitro* duplication and 'cure' of hematopoietic defects in genetically anemic mice. Nature (Lond.), *269:* 412–413, 1977.
4. Dexter, T. M., Allen, T. D., and Lajtha, L. G. Conditions controlling the proliferation of hematopoietic stem cells *in vitro*. J. Cell Physiol., *91:* 335–344, 1977.
5. Zipori, D., and Sasson, T. Adherent cells from mouse bone marrow inhibit the formation of colony stimulating factor (CSF) induced myeloid colonies. Exp. Hematol., *8*: 816–817, 1980.
6. Harigaya, K., Murase, T., Nakamura, M., and Watanabe, Y. Fibrocytic cells derived from human bone marrow cultures inhibit the formation of myeloid colonies. Hematol. Rev. Commun., *1*: 269–276, 1987.
7. Harigaya, I., and Handa, H. Generation of functional clonal cell lines from human bone marrow stroma. Proc. Natl. Acad. Sci. USA, *82:* 3477–3480, 1985.
8. Harigaya, K., Cronkite, E. P., Miller, M. E., and Shadduck, R. K. Murine bone marrow cell line producing colony-stimulating factor. Proc. Natl. Acad. Sci. USA, *78:* 6963–6966, 1981.
9. Zipori, D., Sasson, T., and Friedman, S. Bone marrow resident colony stimulating factor activity (CSA) produced by stromal cell. *In:* S. J. Baum, G. G. Ledney, and S. Thierfelder (eds.), Experimental Hematology Today 1982, pp. 19–26. Basel, New York: Karger, 1982.
10. Collins, S. J., Gallo, R. C., and Gallagher, R. E. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature (Lond.), *270:* 347–349, 1977.
11. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. J. Exp. Med., *149:* 969–974, 1979.
12. Enomoto, T., Martel, N., Kanno, Y., and Yamasaki, H. Inhibition of cell communication between BALB/c 3T3 cells by tumor promoters and protection by cAMP. J. Cell Physiol., *121:* 323–333, 1984.
13. Moore, G. E., Woods, L. K., Minowada, J., and Mitchen, J. R. Establishment of leukemia cell line with T cell characteristics. *In Vitro*, (Rockville), *8:* 434, 1973.
14. Miyoshi I., Hiraki, S., Tsubota, T., Kubonishi, I., Matsuda, Y., Nakayama, T., Kishimoto, H., and Kimura, I. Human B cell, T cell and null cell leukemic cell lines derived from acute lymphocytic leukemias. Nature (Lond.), *267:* 843–844, 1977.
15. Mangelsdorf, D. J., Koeffler, H. P., Donaldson, C. A., Pike, J. W., and Haussler, M. R. 1,25-Dihydroxyvitamin D_3-induced differentiation in human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. J. Cell Biol., *98:* 391–398, 1984.
16. Koeffler, H. P., Amatruda, T., Ikegawa, N., Kobayashi, Y., and Deluca, H. F. Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1,25-Dihydroxyvitamin D_3 and its fluorinated analogues. Cancer Res., *44:* 5624–5628, 1984.
17. Tanaka, H., Abe, E., Miyaura, C., Kuribayashi, T., Konno, K., Nishii, Y., and Suda, T. 1α,25-Dihydroxycholecalciferol and a human myeloid leukemia cell line (HL-60). Biochem. J., *204:* 713–719, 1982.
18. Miyaura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K. Nishii, Y., and Suda, T. 1α,25-Dihydroxyvitamin D_3 induces differentiation of human myeloid leukemia cells. Biochem. Biophys. Res. Commun., *102:* 937–943, 1981.
19. Flynn, P. J., Miller, W. J., Weisdorf, D. J., Arthur, D. C., Brunning, R., and Bronda, R. F. Retinoic acid treatment of acute promyelocytic leukemia: *In vitro* and *in vivo* observations. Blood, *62:* 1211–1217, 1983.