Induction of Differentiation of HL-60 Cells by the Anti-fungal Antibiotic, Radicicol

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The anti-fungal antibiotic, radicicol, produced in the culture broth of *Neocosmospora tenuicristata*, was found to induce differentiation of HL-60 cells into macrophages from the following evidence: (1) it caused morphological changes into macrophage-like cells, (2) induced NBT (Nitrobluetetrazolium) reduction activity, (3) induced phagocytosis, and (4) induced α-naphthyl acetate esterase activity. The concentration of radicicol required to differentiate HL-60 cells is 50 ~ 100 ng/ml, and the incubation time required for commitment of differentiation is 16 hours. Flow cytometry analysis indicated that radicicol blocks the cell cycle of HL-60 cells at the G1 and G2 sites. In addition, radicicol induced reversal of the transformed phenotype of ras-transformed NIH3T3 cells (DT cells) at 25 ng/ml.

In vitro studies have shown that certain types of cultured tumor cells, such as leukemic, neuroblastoma, and melanoma cells, can be differentiated and their proliferation ceased by treatment with various differentiation inducers[1,2]. These include a kind of hormone[3], vitamine[4,5], polas compound[6], and modulators of oncogene functions[7,8]. Among them, potent inducers such as 1, 25$(OH)_2$ vitamine D_3 or dexamethasone were reported to exert antitumor activity in mice inoculated with myeloid leukemic cells[9,10].

These findings increased the possibility that induction of differentiation of tumor cells into normal cells is a new type of therapy for human cancer. It was reported by several investigators that low doses of cytosine arabinoside (Ara-C), which were sufficient to induce differentiation but not cytotoxic effects, are clinically available for myeloid leukemia patients[11]. HUANG *et al.*[12] and CASTAIGNE *et al.*[13] showed that all-*trans* retinoic acid, a potent differentiation inducer for leukemic cells *in vitro*[5], is also remarkably effective in attaining complete remission of acute promyelocytic leukemia patients.

With the aim of developing new cancer therapy drugs, we have screened differentiation-inducing compounds from microbial metabolites using human promyelocytic leukemia cells HL-60[14]. In this paper, we report that an anti-fungal antibiotic, radicicol (Fig. 1)[15~17], induces the differentiation of HL-60 into macrophage-like cells.

Materials and Methods

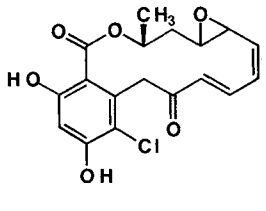
Purification of Radicicol

Radicicol[15~17] was produced in the culture broth of *Neocosmospora tenuicristata* cultivated in a 30-liter jar containing 15 liters of 5% glycerine, 5% potato homogenate, 5% malt extract, and 0.5% yeast extract, pH 6.0, at 26°C for 6 days. Radicicol was extracted at neutral pH, crystallized in ethyl acetate solution, and further purified by chromatography on an LH-20 column eluted by ethyl acetate - methylene chloride (1 : 1), giving 450 mg of radicicol with 98% purity.

Cell Lines and Culture

HL-60 cells were kindly provided by Dr. R. C. GALLO (National Cancer Institute, U.S.A.). DT cells and B16BL6 cells were provided by Dr. M. NODA and Dr. T. TSURUO (Cancer Institute, Japan), respectively. ZR-75-1, L929 and Chinese hamster ovary (CHO) cells were purchased from Dainippon Pharmaceutical Co. Ltd. (Japan). HL-60 cells were cultured in RPMI-1640 medium (Nissui, Japan) containing 1% fetal calf serum (FCS, GIBCO) and 10% GIT medium (Wako Pure

Fig. 1. Structure of radicicol.



Chemical Industries, Japan). DT cells and L929 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) containing 5% FCS. ZR-75-1 cells were cultured in RPMI-1640 medium containing 10% FCS. CHO cells and B16BL6 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS. One hundred U/ml penicillin G, 100 μg/ml streptomycin and 1.2 mg/ml $NaHCO_3$ were added to the media used in this study. All the incubations were carried out in a humidified 5% CO_2 and 95% air atmosphere at 37°C.

Viable cell numbers of HL-60 and trypsinized ZR-75-1 were assessed by trypan blue dye exclusion tests. Viabilities of the other cell lines were judged by 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay[18]. Briefly, cells cultured in 96 multi-well plates in the presence of test compounds, received 50 μl of 1 mg/ml of MTT solution and were then incubated at 37°C for 4 hours. After the medium was discarded, the cells were dissolved in dimethylsulfoxide (DMSO), and the absorbance at 540 nm was measured.

Assays for Differentiation Markers of HL-60 Cells

Unless otherwise described, 2.5×10^5 of HL-60 cells were plated into 24 multi-well plates (Corning) containing 1 ml of medium, and incubated for 72 hours in the presence of appropriate concentrations of test compounds. The cells harvested from each well were pelleted by centrifugation at $150 \times g$ for 3 minutes, and washed once with phosphate-buffered saline (PBS). For determining nitroblue tetrazolium (NBT) reduction activity[19], cells were resuspended in 200 μl of RPMI-1640 medium containing 1 mg/ml of NBT and 100 ng/ml of 12-*O*-tetradecanoylphorbol 13-acetate (TPA). After incubation at 37°C for 20 minutes, aliquots were taken to determine the percentage of cells with formazan deposits under light microscopy. The residual cells were pelleted and resolved in 750 μl of DMSO, and their absorbance at 572 nm was determined.

Phagocytic activity was determined according to the method of COLLINS *et al.*[20]. Briefly, HL-60 cells were washed once with PBS and resuspended in 1 ml of culture medium containing 10% fresh human serum and 4×10^6 particles of heat-treated *Candida albicans*. After incubation at 37°C for 2 hours, the number of HL-60 cells which phagocyted more than 3 particles of Cabdida, was counted.

Nonspecific esterase activity was determined with a commercially available kit (Sigma).

Assays for Differentiation Markers of B16BL6 Cells and ZR-75-1 Cells

B16BL6 cells were plated into 96 multi-well plates (Corning) at a density of 4×10^3 cells/well with 100 μl of medium. After 24 hours incubation, the medium was replaced with the test medium with or without inducers, and cells were incubated for a further 120 hours. At the end of the incubation, medium from each well was transferred into an empty plate. After the cells in the well were dissolved with 50 μl of DMSO, the cell lysate and medium were pooled and their absorbance at 405 nm was measured for determination of melanin content.

ZR-75-1 cells were plated into 6 multi-well plates (Corning) at a density of 1×10^5 cells/well with 2 ml of medium and cultured for 24 hours. The cells were then incubated for a further 120 hours in the presence or absence of inducers. The cells were scraped from the well by a rubber policeman and suspended in 5 ml of PBS. The cells were pelleted by centrifugation at $150 \times g$ for 5 minutes, and washed once with PBS. Cellular lipid was extracted with 1 ml of isopropyl alcohol, from which the triglyceride content was determined using a Triglyceride G-Test Wako kit (Wako Pure Chemical Industries, Japan).

Flow Cytometry

HL-60 cells fixed and permialized with 80% ethanol were resuspended in solution containing 50 μg/ml of propidium iodide, 0.1% Triton X-100 and 3.8 mM sodium citrate, and incubated at 37°C for 30 minutes. The distribution pattern of the DNA content in the cell cycle was analyzed by flow cytometer (Coulter, EPICS 750), counting 10,000 cells for each assay.

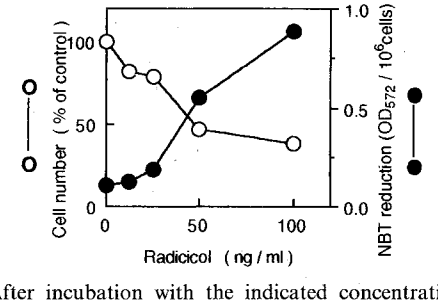
Results

Induction of Differentiation of HL-60 Cells

The culture broth of *Neocosmospora tenuicristata* contained large amounts of a substance which reduced cell growth and increased the NBT reduction activity of HL-60. On purification, the differentiation-inducing compound was identified as radicicol (Fig. 1), an antifungal antibiotic isolated by DELMOTTIE[5].

Radicicol induced NBT reduction activity and reduced the growth of HL-60 cells dose dependently (Fig. 2). The concentration of radicicol required for 50% inhibition of cell growth (I_{50} value) was 50 ng/ml. By addition of 100 ng/ml of radicicol, which had no effect on the viability

Fig. 2. Suppression of cell growth and induction of NBT reduction activity by radicicol in HL-60 cells.



After incubation with the indicated concentrations of radicicol for 72 hours, NBT reduction activity (closed circle, see right vertical axis) and cell numbers (open circle, see left vertical axis) were determined. Each point represents the average of duplicate assays.

of the HL-60 cells, 72% of the whole cell population became NBT reduction-positive, and 98% of the cells became phagocytic (Table 1).

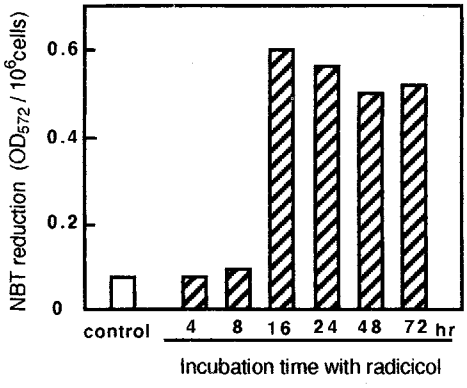
In order to discover the incubation time required for commitment of differentiation by radicicol, HL-60 cells were treated with 100 ng/ml of radicicol for various periods, and their NBT reduction activities were determined at the end of 72-hour incubation. As indicated in Fig. 3, the cells incubated with radicicol for 16 hours and transferred to a radicicol-free medium showed an NBT reducing activity equal to that of the cells treated with radicicol-containing medium throughout 72 hours incubation. Treatment for less than 8 hours caused neither growth inhibition nor differentiation.

Table 1. Expression of differentiation markers in HL-60 cells by radicicol.

Addition	Conc.	Positive cell, %	
		NBT reduction	Phagocytosis
None		2	4
Radicicol	12.5 ng/ml	5	45
	25 ng/ml	19	56
	50 ng/ml	28	87
	100 ng/ml	72	98
Retinoic acid	300 ng/ml	56	95
DMSO	1.3%	76	99

HL-60 cells were incubated for 72 hours with the indicated compounds. Each value represents the average of duplicate assays.

Fig. 3. Induction of NBT reduction activity in HL-60 cells incubated with radicicol for various times.



HL-60 cells which had been incubated with 100 ng/ml of radicicol (hatched bar) for the times indicated below each bar, were transferred into radicicol-free medium, and their NBT reducing activity was determined after 72 hours total incubation. Control cells (open bar) were incubated in the absence of radicicol throughout 72 hours. Each bar represents the average of duplicate assays.

Radicicol induced α-naphthyl esterase activity in HL-60 cells in a dose-dependent manner (Fig. 4). By 50 ng/ml of radicicol, about 30% of cells were induced to express esterase activity. Although this percentage was much lower than that of the NBT reduction-positive or phagocytic cells (Table 1), it was comparable to the potential of 1 ng/ml of TPA which is known to differentiate HL-60 cells along the macrophage lineage[21]. In contrast, retinoic acid, a differentiation inducer towards granulocytes[5], did not induce esterase activity in HL-60 cells.

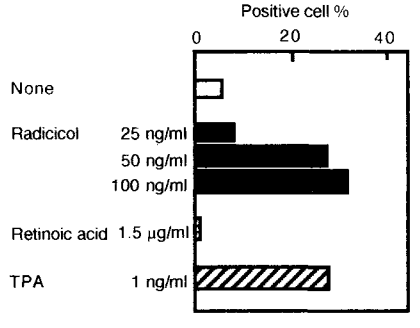
May-Gruenwald, Gimsa staining of HL-60 cells is shown in Fig. 5. Treatment with 100 ng/ml of radicicol for 72 hours caused a remarkable morphological change in the HL-60 cells, inducing typical features of macrophages. This result, together with the induction of α-naphthyl acetate esterase, indicates that radicicol induces HL-60 to differentiate in a macrophage direction. Radicicol, however, hardly increased cell adherence to the substrate.

Effect of Radicicol on Differentiation of B16BL6 and ZR-75-1 Cells

The differentiation-inducing activity of radicicol was also evaluated in mouse melanoma B16BL6 cells and human mammary cancer ZR-75-1 cells (Fig. 6). When B16BL6 cells were incubated with radicicol for 120 hours, growth was reduced with an I_{50} value of 230 ng/ml. However, melanin production, which is a differentiation marker of this cell line[22,23], was not increased by addition of 500 ng/ml of radicicol. On the other hand, dibutyryl cyclic AMP, a known differentiation inducer for this cell line[22,23] and also for HL-60[24], increased melanin production by 4.5 fold over control cells.

In ZR-75-1 cells, although 870 ng/ml of radicicol

Fig. 4. Induction of α-naphthyl acetate esterase activity by radicicol and other differentiation inducers in HL-60 cells.



After HL-60 cells were incubated with the indicated compounds for 72 hours, the percentages of cells expressing esterase activity were determined. Each bar represents the average of duplicate assays.

Fig. 5. Morphology of HL-60 cells stained with May-Grunwald-Giemsa.

(A) Untreated cells, (B) Cells treated with 100 ng/ml of radicicol for 72 hours. Original magnification, ×400.

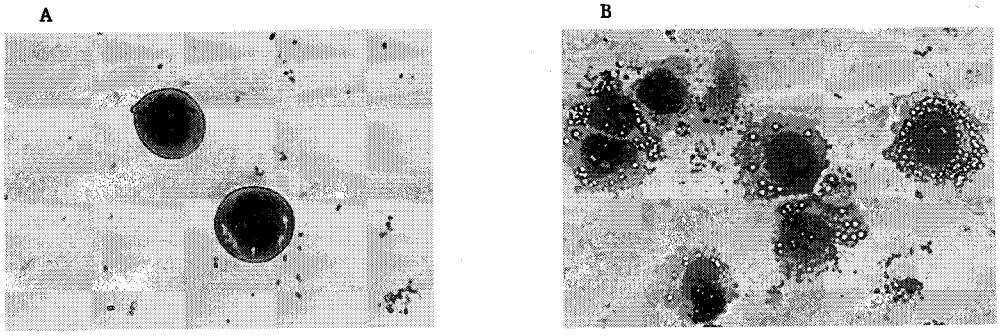
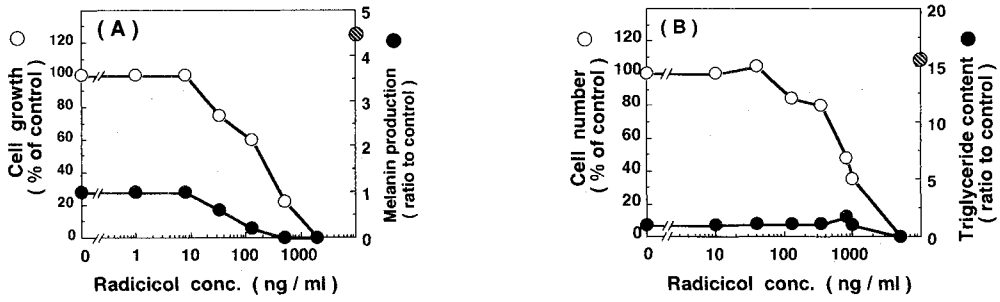


Fig. 6. Effect of radicicol on differentiation of B16BL6 and ZR-75-1 cells.



(A) B16BL6 cells were treated with the indicated concentrations of radicicol for 120 hours and were assessed for cell growth (open circle, see left vertical axis) and melanin production (closed circle, see right vertical axis). Hatched circle expresses the melanin production in cells treated with 0.125 mM dibutyryl-cyclic AMP. Each point represents triple assays.

(B) ZR-75-1 cells were treated with the indicated concentrations of radidicol for 120 hours and were assessed for cell growth (open circle, see left vertical axis) and triglyceride content (closed circle, see right vertical axis). Hatched circle expresses the triglyceride content in cells treated with 1 mM sodium butyrate. Each point represents the average of duplicate assays.

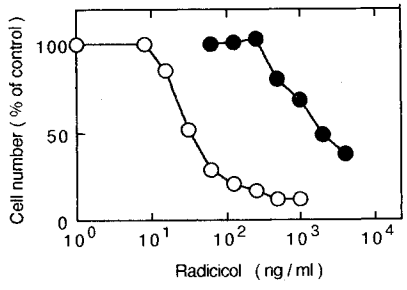
reduced cell growth to 50% of control after 96 hours incubation, it did not induce accumulation of triglyceride in the cells even at a concentation of 1,000 ng/ml. Treating ZR-75-1 cells with sodium butyrate, a differentiation inducer[25], caused a 16-fold accumulation of triglyceride in the cells compared with the control.

Thus, radicicol has no capacity to induce differentiation of B16BL6 or ZR-75-1, suggesting that the differentiation of HL-60 cells by radicicol is not mediated through the accumulation of cyclic AMP.

Kinetics of Growth Inhibition by Radicicol

The susceptibility of HL-60 to radicicol was compared under different cell density conditions (Fig. 7). The cells plated at a low cell density (2.5×10^5 cells/ml), which grew rapidly during 48 hours incubation, were sensitive to growth inhibition of radicicol, giving an I_{50} value of 50 ng/ml. On the other hand, for the cells plated at a high cell density (1.5×10^6 cells/ml), of which proliferation did not proceed further during 48 hours, it required more than 2,000 ng/ml of radicicol to decrease the numbers of viable cells to 50% of the control. The latter action of radicicol may be attributed to cytotoxity. On measuring the amount of radicicol in the medium by

Fig. 7. Effect of radicicol on growth of HL-60 cells plated at low or high cell density.



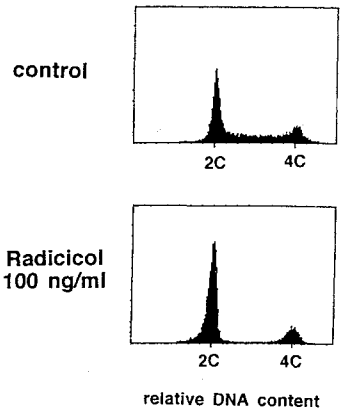
HL-60 cells were plated at a density of 2.5×10^5 cells/well (open circle) or 1.5×10^6 cells/well (closed circle) into 24 well multi-well plates, and the indicated concentrations of radicicol were added. After incubation for 48 hours, the numbers of cells were counted. Each point represents the average of duplicate assays.

HPLC analysis after incubation with the cells, the value obtained in the high cell-density condition was the same as that in the low (data not shown).

Flow cytometry analysis was carried out to find out the inhibition site of radicicol in the cell cycle of growing cells. As shown in the DNA histogram in Fig. 8, accumulation of cells at the 2C and 4C peaks was observed in HL-60 treated with 100 ng/ml of radicicol for 48 hours. The cells distributing between 2C and 4C peaks, which corresponded to the S-phase, were markedly decreased by radicicol. This DNA histogram pattern, together with the lack of appearance of M-phase cells under light microscopy (data not shown), suggests that radicicol inhibits progression from G1 into the S-phase, and also blocks cell cycle at the G2 site.

Radicicol also exerted its growth-inhibition effects on CHO cells and mouse L-929 cells (Fig. 9). In both cell lines, rapidly growing cells, which were plated at a low cell density, were over 10 times more highly susceptible to radicicol compared with cells in the nearly confluent state. Comparisons of the I_{50} values of radicicol in various cell lines in the rapidly-growing state, among which HL-60 was the most sensitive, are shown in Table 2.

Fig. 8. Effect of radicicol on DNA contents of HL-60 cells.



HL-60 cells (2.5×10^5/ml) were incubated in the presence or absence of 100 ng/ml of radicicol for 48 hours, after which the distribution of DNA contents was analyzed by flow cytometry.

Table 2. Comparison of growth inhibitory concentrations of radicicicol in various cell lines.

Cell line	I_{50} value* (ng/ml)
HL-60[a]	50
B16BL6[b]	230
ZR-75-1[b]	870
L929[a]	200
CHO[a]	500

* Concentration of radicicol required for 50% inhibition of cell growth.
[a] Incubation time with radicicol was 72 hours.
[b] Incubation time with radicicol was 120 hours.

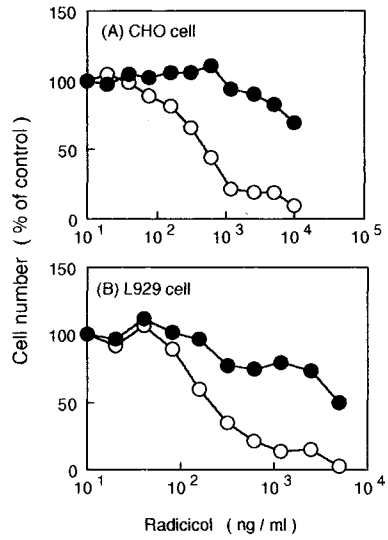
Effect of Radicicol on the Morphology of Ras-transformed Cells

In order to examine the effect of radicicol on oncogene function, DT cells, NIH3T3 cells transformed by the introduction of 2 copies of v-ras[26], were treated with radicicol. The concentration of radicicol required for 50% inhibition of cell growth was 65 ng/ml after 48 hours incubation. As shown in Fig. 10, DT cells incubated in the presence of 25 ng/ml of radicicol for 20 hours became flat like normal NIH3T3 cells, whereas control DT cells exhibited a round shape.

Discussion

It was indicated in this study that an anti-fungal antibiotic, radicicol[15~17], blocks the cell cycle of HL-60 cells at the G1 and G2 sites, and induces irreversible differentiation along the macrophage lineage. These phenomena were observed at 50~100 ng/ml, which is a much lower concentration range than that giving anti-fungal activity, ranging from 3~300 μg/ml[15,17]. Radici-

Fig. 9. Effect of radicicol on cell growth of CHO cells and L929 cells.

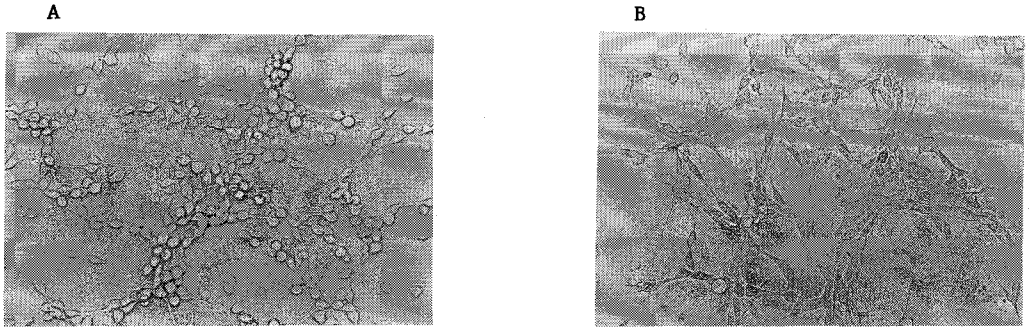


CHO cells (A) were plated into 96 well multi-well plates at a density of 5×10^3 cells/well (open circle) or 5×10^4 cells/well (closed circle).

L929 cells (B) were plated into 96 well multi-well plates at a density of 2×10^3 cells/well (open circle) or 2×10^4 cells/well (closed circle).

After incubation with the indicated concentrations of radicicol for 72 hours, viable cell numbers were determined. Each point represents the average of duplicate assays.

Fig. 10. Morphological changes in DT-cells caused by radicicol.



DT-cells were plated into 24 well multi-well plates at a density of 2×10^4 cells/well and cultured for 24 hours. The cells were then incubated in the (A) absence or (B) presence of 25 ng/ml of radicicol for 20 hours, after which the morphology of the cells was examined by light microscopy. Original magnification, ×400.

col also had cytotoxic activity at high doses equivalent to anti-fungal concentrations. Comparing these concentrations, the differentiation-inducing activity seems to be the most specific action among the various biological activities of radicicol.

Radicicol had no differentiation-inducing effect on ZR-75-1 or B16BL6 cells, in spite of showing growth inhibition, suggesting that no protein kinase A-mediated reaction is involved in the differentiation of HL-60 cells by radicicol.

KWON *et al.* have already reported biological activities of radicicol other than anti-fungal action[27,28]; (1) induction of reversal of the transformed phenotype of both v-src-transformed and c-erbB-2-transformed 3Y1 cells, (2) G1G2 arrest of rat 3Y1 cells, (3) inhibition of tyrosine kinases of both src and receptor types, (4) induction of differentiation of Friend leukemia cells. These phenomena were observed at around 100 ng/ml of radicicol. Differentiation of Friend leukemia cells is already known to be associated with the inhibition of tyrosine kinase[29]. The fact that radicicol also inhibits growth of normal cells such as CHO cells and L929 cells (Fig. 9) may be attributed to the inhibition of tyrosine kinase. Besides, reversal of the transformed phenotype of DT cells by radicicol in our study might result from inhibition of tyrosine kinase, as ras is located downstream of regulation by receptor type tyrosine kinase[30]. Although it should be notable that ras is activated in HL-60 cells together with myc alteration[31], the mechanism of HL-60 differentiation by radicicol, and its relation to tyrosine kinase, ras fuction and G1G2 arrest, must be solved by further work.

The LD_{50} value of radicicol in mouse acute-toxicity tests was between 300 and 1,000 mg/kg by oral administration (our preliminary experiment). In addition to the low toxicity *in vivo*, the fact that radicicol also exhibited cytostatic activity on P388 cells, L1210 cells and M5076 cells at as low as 10 ng/ml *in vitro* (data not shown), prompted us to evaluate the potency of this compound *in vivo*. Anticancer tests of radicicol in tumor-bearing mice are now in progress.

Acknowledgments

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