Actinomycin V as a Potent Differentiation Inducer of F5-5 Friend Leukemia Cells

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The cell differentiation system of Friend leukemia cells was applied to screening for new types of antitumor antibiotics. F5-5, Friend leukemia cells, were the most suitable for the assay system due to the stability of their response on repeated culture passages. Antibiotics like mitomycin C, adriamycin and actinomycin D, but not cycloheximide, did not induce detectable benzidine-positive cells among the F5-5 cells in the concentration ranges tested. Among the culture fluids of one thousand and fifty-one streptomycete strains subjected to the assay system, actinomycin V, FL-518 and FL-657 were found to be the most active as inducers. Actinomycin V possessing L-4-ketoprolin as a substitute for L-proline of actinomycin D at a concentration of 1.0 ng/ml caused 39.7% of the F5-5 cells to become benzidine-positive. Furthermore, actinomycin V inhibited the colony formation of F5-5 cells in the soft agar medium at a concentration of 0.004 ng/ml.

Two kinds of assay system have been developed for screening for new antitumor substance; *in vivo* and *in vitro* systems. The *in vivo* systems were effectively adopted to test the antitumor activity of newly found or synthesized compounds using established tumor cells. However, the discovery of an new antitumor compound of microbial origin needs an *in vitro* antitumor assay system.^{1~2)} Microorganisms excrete diverse metabolites including antitumor compounds. Due to their extremely low amounts in culture fluids, on *in vivo* antitumor assay system does not always detect their antitumor activity.

An *in vitro* detection system for antitumor activity usually depends on the cytotoxic effect on tumor cells, making it difficult to find an effective antitumor compound with selective toxicity. Therefore, we applied the differentiation induction system of Friend leukemia cells to discover antitumor compounds having selective action on tumor cells. Erythroleukemia cells can differentiate into hemoglobin-positive normal erythroblast-like cells on chemical stimulation.^{3~6)} These chemicals include bleomycin, adriamycin and actino-

mycin D, which are used clinically.^{7,8)}

This paper deals with application of the differentiation induction system of Friend leukemia cells, F5-5, to the culture fluids of 1051 strains of streptomycetes. Consequently, we found three potent inducers, one of which was actinomycin V.

MATERIALS AND METHODS

Cell lines and culture conditions. Friend leukemia cell lines, F5-5 and C9-6, were gifts from Dr. Ikawa, who established them from DDD mice.⁹⁾ F5-5 differentiated to biosynthesize hemoglobin on induction by DMSO, but C9-6 not. Another Friend leukemia cell line, C1745A, originated from DBA/2 mice³⁾ was donated by Dr. Onodera. The culture media were Ham's F-12 supplemented with 10% fetal calf serum for F5-5 and C9-6, and Dulbecco's MEM supplemented with 10% fetal calf serum for C1745A. Cells were cultured at 37°C in a 5% CO₂-95% air incubator.

96-well plates were used for the assay. The initial cell densities were 2×10^4 cells/ml for F5-5 and C9-6, and 1×10^5 cells/ml for C1745A. The Culture volume was 0.2 ml of which 0.02 ml was the microbial culture fluid to be tested or a dilution of it.

Viable cells were determined by the trypan blue dye exclusion method and counted with a Burker-Turk counter.

To determine the proportion of hemoglobin-containing cells, 0.2 ml of a cell suspension was mixed with 20 μ l of a freshly prepared benzidine solution (10 to 1 mixture of 2% 3,3'-dimethoxy benzidine in 0.5M acetic acid and 30% hydrogen peroxide).¹⁰⁾ Cells that were stained blue, indicating the presence of hemoglobin, were counted in a hemacytometer and expressed as the percent of the total cell number.

In order to examine cell tumorigenicity, a colony formation assay was carried out as follows¹¹⁾: F5-5 cells were suspended in Ham's F-12 medium supplemented with 10% fetal calf serum to a cell density of 2×10^3 cells/ml. The cell suspension (4 ml) was mixed with 16 ml of an agar medium consisting of 0.35 ml of 7.5% NaHCO₃, 11.25 ml of Ham's F-12 medium concentrated two times, 2.5 ml of fetal calf serum, 3.75 ml of 3% agar (Difco) and 7.15 ml of water. The above cell suspension (0.5 ml) was overlaid on 0.5 ml of the agar medium in a 24-well plate. One ml of the liquid medium containing 20% a samples was placed on the solidified medium. The agar culture was carried out at 37°C for 10~14 days in a 5% CO₂-95% air incubator, and the colonies that formed were counted.

Culture conditions for streptomycetes and preparation of culture filtrates. The culture medium was composed of 2% soluble starch, 1% glucose, 0.2% yeast extract, 0.1% KH₂PO₄, 0.1% MgSO₄·7H₂O, 0.7% bacto-soytone and 0.5% corn steep liquor. The pH was adjusted to 7.0 with NaOH. Streptomycetes were cultured at 27°C for 7 days in 100 ml of medium in 500 ml Erlenmeyer flasks. The supernatants were sterilized with filter papers (pore size, 0.45 μ m), and subjected to the assay.

Materials. Actinomycin V, actinomycin D, vinblastine, puromycin aminonucleoside, rifamycin SV, valinomycin, cycloheximide, antimycin, erythromycin and azaserine were obtained from Sigma, aureomycin, bacitracin and distamycin A from Serva, mitomycin C from Boehringer Mannheim, daunomycin from Meiji Seika Co., Ltd., and adriamycin from Kyowa Hakko Co., Ltd.

Abbreviation. DMSO, dimethyl sulfoxide.

RESULTS

Cells employed for the assay system

Two kinds of Friend leukemia cell lines, F5-5 and C1745A, were comparatively studied in relation to their mode of response to induction by DMSO and the stability of their differentiation potency after repeated passages. Table I shows that approximately 1% of the C1745A cells differentiated in the absence of DMSO, however, the F5-5 cells did not at all. This

TABLE I. DIFFERENTIATION OF FRIEND LEUKEMIA CELLS INDUCED BY DMSO

Friend leukemia cells were cultured in the presence of 1.5% DMSO for 7 days.

Cell line	DMSO (1.5%)	Benzidinepositive cells per total cells	(%)
F5-5	—	0/255, 0/240	0
	+	98/197, 83/194	46.3
C1745A	—	1/105, 1/124	0.9
	+	68/108, 54/98	59.0

TABLE II. STABILITY OF THE SENSITIVITY OF F5-5 AND C1745A CELLS TO INDUCTION BY DMSO

The cultures of F5-5 and C1745A were transferred twice a week. After the passage times indicated, the ability of the cells to differentiate was determined. Benzidine-positive cells were scored after the cultivation times indicated in the presence of 1.5% DMSO.

Cell line	Passage	Cultivation (days)	Benzidine-positive cells (%)
F5-5	1	5	34.0
		6	69.3
		7	71.2
		8	72.0
		22	64.0
C1745A	1	4	52.7
		6	83.2
		7	63.0
		8	26.0
		20	40.1

suggests that a part of the C1745A cells spontaneously differentiated into hemoglobin-positive cells. Therefore, it was found to be preferable to use F5-5 in a search for new differentiation inducing agents.

For both F5-5 and C1745A, 50~70% of the cells differentiated. This property of F5-5 was continuously kept after repeated passages for 22 times, but not in the case of C1745A, as shown in Table II. The heterogeneity of the C1745A cells seemed to cause the unsteadiness of the response of C1745A to DMSO. In addition, antibiotics such as actinomycin D, mitomycin C and adriamycin did not cause differentiation of F5-5 (Table IV).^{7,8)} Because of the above mentioned data, F5-5

FIG. 1. Rate of Appearance of Benzidine-positive Cells in Culture with DMSO.

○—○, viable cells and ○---○, benzidine-positive cells in culture with 1.5% DMSO; ●—●, viable cells and ●---●, benzidine-positive cells in a control culture.

was employed for the screening assay.

The optimal conditions for differentiation of F5-5 cells were studied in the presence of 1.5% DMSO (Fig. 1 and Table III). The cells at initial concentrations of 2.5 and 5.0×10^4 cells/ml showed their maximal growth (7×10^5 cells/ml) at 115 hr. The maximal ratio of benzidine positive cells was observed at 140 hr (Fig. 1).

Initial cell densities of more than 5×10^4 cells/ml did not allow sufficient cell differentiation. Therefore, the initial cell density of approximately 2.5×10^4 cells/ml and an incubation time of 6 days were taken as the optimum conditions (Table III).

FIG. 2. Effects of DMSO on Hemoglobin Biosynthesis and Colony Formation in the Soft Agar Medium.

Relative survival of colonies in the soft agar medium was determined as colonies in the presence of the agent per those in the control culture. —●—, viable cells in the liquid medium; —○—, benzidine-positive cells; —△—, relative surviving colonies in the soft agar medium.

TABLE III. EFFECT OF INITIAL CELL DENSITY ON CELL GROWTH AND DIFFERENTIATION OF F5-5

At the times indicated, 0.2 ml of the culture was taken out from 10 ml of the medium containing 1.5% DMSO in a 25 ml culture flask. Viable and benzidine-positive cells were scored at the times indicated.

Initial cell density ($\times 10^4$ cells/ml)	Viable cells ($\times 10^4$ cells/ml)				Benzidine-positive cells (%)			
	3	4	5	6	3	4	5	6
	(days)				(days)			
2.5	24	48	75	55	0	0	29.0	52.5
5.0	49	95	75	46	0	0	32.5	51.0
10.0	73	81	60	41	0	0	12.5	14.2
25.0	83	65	41	27	0	0	2.5	0

TABLE IV. ABILITIES OF VARIOUS ANTIBIOTICS TO INDUCE BENZIDINE-POSITIVE CELLS AND INHIBIT CELL GROWTH OF F5-5 IN THE LIQUID MEDIUM

All antibiotics were incubated with the F5-5 cells in the concentration ranges indicated for 6 days. Effective doses of agents (ED_{50}) giving half maximal growth were calculated from the dose-response curves for the compounds.

Antibiotics	Concentration ranges ($\mu\text{g/ml}$)	ED_{50} ($\mu\text{g/ml}$)	Benzidine-positive cells
DNA synthesis inhibitor			
Mitomycin C	$10^{-6} \sim 10^0$	5×10^{-1}	—
RNA synthesis inhibitors			
Actinomycin D	$10^{-6} \sim 10^0$	5×10^{-2}	—
Daunomycin	$10^{-5} \sim 2 \times 10^1$	10^{-3}	—
Adriamycin	$10^{-6} \sim 10^0$	5×10^{-3}	—
Rifamycin SV	$5 \times 10^{-5} \sim 5 \times 10^{-4}$	$> 5 \times 10^{-4}$	—
Distamycin A	$10^{-6} \sim 10^0$	$> 10^0$	—
Protein synthesis inhibitors			
Puromycin aminonucleoside	$5 \times 10^{-5} \sim 5 \times 10^{-4}$	$> 5 \times 10^{-4}$	—
Erythromycin	$10^{-6} \sim 10^0$	$> 10^0$	—
Cycloheximide	$2 \times 10^{-2} \sim 4 \times 10^{-1}$	5×10^{-1}	+
Energy transfer inhibitors			
Antimycin A	$10^{-6} \sim 10^0$	$> 10^0$	—
Valinomycin	$10^{-10} \sim 10^{-3}$	5×10^{-5}	—
Nucleic acid synthesis inhibitor			
Azaserine	$10^{-6} \sim 10^0$	10^0	—
Membrane and mitosis inhibitors			
Bacitracin	$10^{-6} \sim 10^0$	$> 10^0$	—
Vinblastine	$10^{-6} \sim 10^0$	5×10^{-3}	—

+, benzidine-positive cells observed; —, no benzidine-positive cells observed.

FIG. 3. Effects of Actinomycin D, Adriamycin and Mitomycin C on Viability in the Liquid Medium and Colony Formation in the Soft Agar Medium of F5-5 Cells.

Relative viable cells and colonies in the presence of agents were calculated by comparison with those in the control culture. —●—, relative viable cells in the liquid medium; —○—, relative surviving colonies in the soft agar medium.

The loss of the ability to form colonies in the soft agar medium was followed by hemoglobin biosynthesis as shown in Fig. 2. Colony formation in the soft agar medium was markedly inhibited by 1% DMSO, but cell growth in the

liquid medium was not.

Effect of antibiotics on cell differentiation

Several kinds of antibiotics were examined as to their ability to induce hemoglobin bio-

synthesis in F5-5 cells. They included mitomycin C, a DNA synthesis inhibitor, actinomycin D, daunomycin, adriamycin, rifamycin SV and distamycin A, RNA synthesis inhibitors, puromycin, erythromycin and cycloheximide, protein synthesis inhibitors, antimycin A and valinomycin, energy transfer inhibitors, and azaserine, bacitracin and vinblastine.¹²⁾ Antibiotics other than cycloheximide did not induce hemoglobin biosynthesis in F5-5 cells in the concentration ranges tested (Table IV).

Actinomycin D, adriamycin and mitomycin C inhibited cell growth of F5-5 cells in the soft agar and liquid media (Fig. 3). This suggests that these antibiotics nonspecifically inhibit the growth of F5-5.

Application to culture fluids of streptomycetes

Samples of culture fluids of one thousand and fifty-one streptomycete strains were subjected to the assay system. Among them, 22 samples showed activity toward F5-5 cells. These samples were examined as to their activity not only toward F5-5 but also toward C9-6 and C1745A (Table V). Two of them, FL-1022 and FL-2371, did not show activity toward C9-6 at a concentration of 10%, but the other twenty samples showed activity toward all three kinds of Friend leukemia cells.

TABLE V. MINIMUM DOSES OF CULTURE FLUIDS OF STREPTOMYCES CAUSING F5-5 CELLS TO BECOME BENZIDINE-POSITIVE

Culture fluids sterilized with membrane filters (pore size, 0.45 m μ) were added to the cell culture medium at a concentration of 10%. The minimum dose of a sample was expressed as the concentration inducing at least 1% benzidine positive F5-5 cells.

Samples	Minimum doses ($\times 10^{-2}\%$)		
	F5-5	C9-6	C1745A
FL- 53	1.95	0.98	62.5
FL-124	7.8	1.95	250
FL-178	62.5	0.12	62.5
FL-198	7.8	0.98	125
FL-300	0.98	0.98	15.6
FL-514	1.95	0.12	250
FL-518	0.49	0.24	0.98
FL-602	0.98	0.49	31.3
FL-657	7.8	7.8	15.6
FL-928	15.6	1.95	7.8
FL-977	62.5	62.5	62.5
FL-1022	62.5	NI	62.5
FL-1119	500	500	1000
FL-1189	62.5	31.3	7.8
FL-1208	15.6	15.6	31.3
FL-1448	1.98	0.98	125
FL-1454	62.5	62.5	7.8
FL-1547	62.5	125	62.5
FL-2371	7.8	NI	62.5
FL-2425	7.8	3.9	500
FL-2454	7.8	7.8	7.8

NI, not induced at 10%.

FIG. 4. Chemical Structures of Actinomycins V and D.

Among the twenty samples, FL-518, FL-602 and FL-657 were selected for further studies due to their strong activity and reproducible microbial production. The chemical and biological properties of FL-518 and FL-657 will be described elsewhere.

Actinomycin V as a potent differentiation inducer

One active inducer (FL-602) that accumulated in the culture fluid of *Streptomyces*

venezuelae was identified as actinomycin V by analyses of the UV, IR and ^1H - and ^{13}C -NMR spectra, compared with an authentic sample of actinomycin V.¹³⁾

Actinomycin V is structurally different from actinomycin D, possessing L-4-ketoproline as a substitute for L-proline of actinomycin D (Fig. 4).

The effects of actinomycin V on cell growth and hemoglobin biosynthesis of F5-5 were compared with those of actinomycin D (Table

TABLE VI. EFFECTS OF ACTINOMYCINS V AND D ON DIFFERENTIATION AND CELL GROWTH OF F5-5

Compounds	Conc. (ng/ml)	Viable cells ($\times 10^4$ cells/ml)			Benzidine-positive cells (%)		
		F5-5	C9-6	C1745A	F5-5	C9-6	C1745A
Actinomycin V	0	54.7	45.0	171	0	0	0.9
	0.01	58.6	53.8	232	1.6	0	0.9
	0.1	39.0	60.2	226	19.8	4.8	1.0
	1.0	10.8	37.1	222	39.7	9.5	2.6
	10.0	0	0	143	ND	ND	23.7
Actinomycin D	0.1	58.8	50.9	256	0	0	1.1
	1.0	57.8	49.3	225	0	0.6	0.8
	5.0	15.1	33.1	147	0.9	1.7	6.8
	10.0	2.0	2.7	207	0	2.8	22.4

ND, not determined.

FIG. 5. Effect of Concentrations of Actinomycins V and D on the Cell Growth in the Liquid and Soft Agar Medium and Induction of Benzidine-positive Cells.

Relative viable cells and colonies were calculated by the same method as described in Fig. 3. (A), actinomycin V; (B), actinomycin D; —□—, relative viable cells in the liquid medium; —○—, relative surviving colonies in the soft agar medium; —●—, benzidine-positive cells.

6). Most noticeable was that actinomycin V at a concentration of 1.0 ng/ml caused 39.7% of the F5-5 cells to become benzidine-positive. The induction by actinomycin D was very slight, under 1% benzidine-positive cells being produced at the concentration tested, 5.0 ng/ml. Actinomycin V at a concentration of 1.0 ng/ml induced differentiation of 9.5% of the C9-6 cells, however, actinomycin D only induced 2.8% at a ten times higher concentration than that of actinomycin V. The C1745A cells showed approximately 23% differentiation on induction by the two antibiotics at a concentration of 10.0 ng/ml.

As shown in Fig. 5, actinomycin V also influenced the cell growth of F5-5 cells in the soft agar medium. The half maximal concentrations of actinomycin V for reduction of the colony formation in the soft agar medium and the cell growth in the liquid medium were approximately 0.004 ng/ml and 0.3 ng/ml, respectively, and those of actinomycin D approximately 0.4 ng/ml and 4.0 ng/ml, respectively.

DISCUSSION

Differentiation inducing agents for Friend leukemia cells affect more than two different sites on the plasma membrane and DNA, as reported by Nomura and Oishi.¹⁴⁾ Actinomycins V and D showed cytotoxic effects on F5-5 cells. This suggests that actinomycin V may have a similar DNA binding property to actinomycin D.¹⁵⁻¹⁸⁾

F5-5 cells could be induced to become benzidine-positive by actinomycin V. C9-6, which is a derivative from the same mouse strain as for F5-5, was weakly sensitive to the induction by both actinomycins V and D. Sugano *et al.* reported that T-3-C1-1, the parent cell line of F5-5 and C9-6, treated with actinomycin D was completely negative on benzidine staining.¹⁹⁾ These results suggest that in F5-5 the specificity of inducer-sensitive sites in DNA be retained to recognize the difference between L-4-ketoproline in actinomycin V and L-proline in actinomycin D,

however, C9-6 may have weakened specificity. The difference between actinomycins V and D as to the binding interactions on the DNA-binding sites of F5-5 cells may reflect the specific portions related to cell differentiation.

As for C1745A cells, on the contrary, the affinity of the DNA-binding sites related to the cell differentiation was high enough to permit binding of actinomycins V and D and induction of hemoglobin biosynthesis, as reported by Terada *et al.*⁸⁾

The differentiated Friend leukemia cells lost the colony forming ability in the soft agar medium and simultaneously failed to cause leukemia in the syngeneic hosts, as reported by Sugano *et al.*²⁰⁾ Actinomycin V also inhibited colony formation of F5-5 cells in the soft agar medium at approximately 75 ~ 250 times lower concentrations than those in the case of the liquid medium, however, actinomycin D showed inhibition at a 10 times lower concentration. These results suggest that actinomycin V may cause the specific inducer-mediated cessation of neoplastic growth, however, actinomycin D may cause nonspecific growth inhibition. From the viewpoint of practical use, actinomycin V is superior to actinomycin D for treating mouse leukemias caused by Friend leukemia cells.

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