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ISOLATION AND CHARACTERIZATION OF HIGH AND LOW DIFFERENTIATION-INDUCIBLE FRIEND LEUKEMIA LINES*1

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Induction of erythrodifferentiation was carried out by addition of dimethyl sulfoxide or butyric acid to the culture medium among 80 subclones of T-3-Cl-2 Friend mouse leukemia line. Hemoglobin-positive cells were counted on day 5 of the inducer treatment on the hemocytometer after benzidine staining. Some cell lines were inducible by both of the inducers, but others were insensitive to dimethyl sulfoxide, but sensitive to butyric acid. Eight characteristic T-3-Cl-2 subclones have been selected; 2 highly inducible, 2 moderately inducible, and 4 minimally inducible. The data of differentiation inducibility together with the history of the T-3-Cl-2 line may facilitate extensive application of the T-3-Cl-2 system.

Investigation on differentiation-inducible leukemia cells is considered important in the understanding of leukemic or neoplastic state. As to the cell lines derived from Friend virus-induced leukemia, several ascites lines derived from Friend virus-induced erythroid leukemia showed an erythroid morphology,[9] and also unusually high heme synthesis.[18] Most of cultured lines of Friend virus-induced leukemia in mice are known to be inducible to differentiate towards erythrocytes.[3,8,14] A Friend leukemia line can be induced to differentiate into normoblastoid cells in the soft agar medium,[15] and cells of another Friend leukemia line differentiate into irregular-shaped erythrocytes in a Millipore membrane-sealed diffusion chamber placed in the peritoneal cavity of the syngeneic hosts.[4,8] A cloned line is induced to release erythrocyte-like corpuscles *in vitro* by a budding process.[6]

Dimethyl sulfoxide induces *in vitro* erythrodifferentiation of most of Friend leukemia cell lines.[3,8,14] Other polar compounds have been introduced as potent inducers.[20] Butyric acid was found as another potent inducer in an experiment to test metabolites of cyclic dibutyryl-AMP[11] and in an attempt to clarify effective components of a human placental extract preparation.[5,19]

One of the present authors (Y.I.) previously reported a possible molecular mechanism of erythroid differentiation of Friend leukemia cells, and suggested transcriptional activation of globin genes during induction of differentiation.[6,7,17]

To analyze further the regulatory mechanism of globin gene expression in Friend leukemia cells, phenotypic variants were considered advantageous for a comparative study. Thus, 80 phenotypic variants have been cloned from our clonal T-3-Cl-2 Friend leukemia

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cell line, establishing 80 subclones. One-third of them have been passaged twice weekly over 1 year and have been characterized in detail. They are currently in their 100th to 120th subculture generation.

Since the request of the T-3-Cl-2 line and its subclones has become frequent these days, the briefing of their history and the description of inducibility of differentiation by dimethyl sulfoxide or butyric acid among the selected, most useful subclones are considered helpful in facilitating the scientists in this field to the application of our T-3-Cl-2 system.

Materials and Methods

T-3-Cl-2 Cells: The historical backgrounds of T-3-Cl-2 cells are summarized in Chart 1. SFAT-3 ascites Friend leukemia cells originated from a splenic lesion of Friend virus-induced leukemia in a DDD mouse,[9] and showed a high heme synthesis.[18] SFAT-3 cells were cultured *in vitro* to initiate TSFAT-3 cell line. TSFAT-3 cells were cloned in the soft agar medium according to the method described by Patuleia and Friend,[15] establishing T-3-Cl-1 and T-3-Cl-2 clones.[8] The latter clone is near-tetraploid, and is quite sensitive to dimethyl sulfoxide-induction of erythroid differentiation, and has been used extensively in the study of transcriptional activation of globin genes.

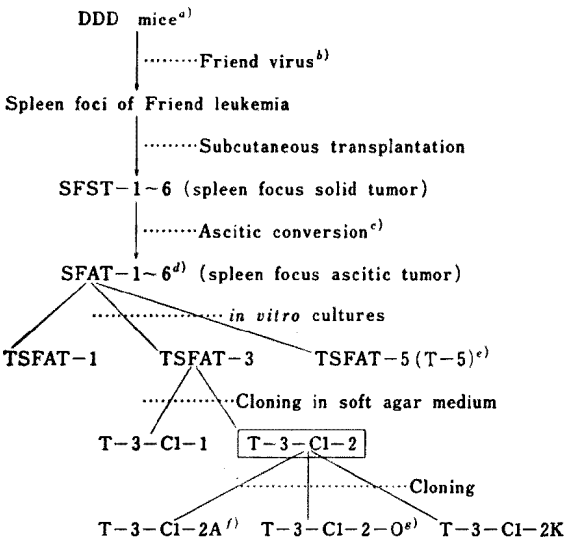

Chart 1. History of T-3-Cl-2 cell

a) Inbred white mouse strain, maintained in the Institute of Medical Science, University of Tokyo.
b) Lyophilized sample of polycythemic Friend virus strain, sent from Dr. C. Friend in 1960.
c) In 1966.
d) High heme synthesis in SFAT-3 and -6 cells.
e) Readily transplantable to syngeneic hosts.
f) More than 80 phenotypic variants were cloned in 1974~1975.
g) Super-inducible clone isolated by S. Orkin.

These Friend leukemia lines have been passaged twice weekly in the Ham F-12 medium (GIBCO) supplemented with 10% heat-inactivated calf serum and 60 mg/liter Kanamycin (Banyu Pharmaceutical Co., Tokyo). The cells have been grown from the concentration of 2.5×10^4 to that of 5×10^5/ml for 4 days. Ten ml of the medium containing 2.5×10^4 cells was provided in a plastic flask of 50 ml (#3013, Falcon Plastics, Oxnard, U.S.A.) to be incubated at 37° with 5% CO_2 flow in a humidified incubator.

Cloning of T-3-Cl-2 Cells: Cells of T-3-Cl-2A, a T-3-Cl-2 subclone (see Chart 1), was readily recloned in microtiter plates (Microtest II, Falcon Plastics). T-3-Cl-2A cells showed a 50~60% cloning efficiency. Approximately 80 subclones have been isolated and characterized in their differentiation inducibility and virus release.[5,10] Differentiation inducibility was expressed in percentage hemoglobin-positive cells by a modified Orkin's technique of benzidine staining.[13]

Eight characteristic T-3-Cl-2 subclones are chosen, which are considered to be most useful for the study of induced differentiation of cultured leukemia cells (Table I). The stability of these subclones has been examined by their differentiation inducibility after a long passage, and by that in the recloned lines.[6]

Benzidine Staining: The hemoglobin-positive cells were examined by a modified Orkin's technique of benzidine staining.[13] The cells were suspended in 0.5 ml of the culture medium, 5 drops of freshly prepared staining solution (3% benzidine in 90% acetic acid:H_2O:30% H_2O_2=10:50:1) were added and, 5~15 min later, the positive cells with bluish black benzidine complex crystals on the cell surface were counted on the hemocytometer. Washing of the cells in the medium or the erythrocyte lysate-added medium did not alter the percentage of hemoglobin-positive cells.

Results

The results are summarized in Table I. T-3-Cl-2 subclones such as C-10-6, C-10-16, F-5-5, and F-5-15 are susceptible to the induction by both dimethyl sulfoxide and butyric acid, and other subclones, such as K-1, C-9-6, and C-9-9, are resistant to dimethyl sulfoxide but

Table I. T-3-Cl-2 and Differentiation Inducibility

Subclones		C-10-6	F-5-5	C-10-16	F-5-15	A-10-10	K-1[a]	C-9-6	C-9-9	(TSFAT-3)
Subculture generation		90	60	90	60	90	120	60	60	650
Control		0.56 (0.2~1.1)	0.02 (0.0~0.04)	0.16 (0.02~0.2)	0.02 (0.0~0.05)	0.00 (0.1~0.6)	0.00	0.00	0.00	0.01 (0.0~0.04)
Dimethyl sulfoxide	1.5% (300m*M*)	51.5 (41~67)	44.8 (37~67)	21.1 (11~43)	10.7 (8.8~19)	0.4 (0.1~0.6)	0.19 (0.0~0.6)	0.07 (0.03~0.1)	0.16 (0.01~0.4)	56.6 (35~72)
	1.7% (340m*M*)	51.4 (42~67)	44.0 (31~50)	22.6 (11~41)	15.0 (9.4~22)	0.26 (0.0~0.5)	0.3 (0.0~0.7)	0.28 (0.04~0.8)	0.94 (0.1~2.3)	58.8 (43~70)
Butyric acid	1.0m*M*	54.1 (40~82)	27.5 (20~37)	21.6 (17~32)	8.4 (4.7~16)	12.4 (6.8~17)	1.3 (0.6~2.8)	18.1 (11~28)	16.0 (9.2~22)	4.3 (1.8~7.9)
	1.25m*M*	61.2 (48~73)	35.5 (29~42)	29.3 (16~47)	11.5 (5.2~24)	16.0 (10~29)	6.3 (3.4~11)	22.2 (13~28)	20.1 (10~23)	4.2 (0.4~9.7)

These subclones were cloned in microtiter plates (cloning efficiency: 50~60%). Differentiation inducibility is expressed as hemoglobin-positive cells (%) on day 5, examined by benzidine staining (range).

a) Still producing spleen focus-forming virus.

sensitive to butyric acid. Among minimally inducible subclones, A-10-10 and K-1 clones are not so stable, whereas C-9-6 and C-9-9 clones are very stable after a long passage.

The parental T-3-Cl-2A line shows differentiation inducibility close to those of C-10-16 and F-5-15, moderate responders. The average differentiation inducibility of T-3-Cl-2A cells on day 5 of treatment with dimethyl sulfoxide has ranged between 25 and 40% in their 300 to 400 subculture generations. The original, uncloned TSFAT-3 cells at their 650 generations are of great interest, because they are sensitive to dimethyl sulfoxide but rather resistant to butyric acid (Table I).

Most of these clones are propagating Friend virus minimally, but K-1 clone is a moderate producer of the spleen focus-forming virus (SFFV), a component of Friend virus complex, polycythemic strain, (SFFV+LLV; lymphocytic leukemia virus[2]). SFFV transforms erythroid precursors. One ml of the 2-day culture medium of 1×10^5/ml of K-1 cells had 40~50 spleen focus-forming activity.[1]

These T-3-Cl-2 subclones, as listed in Table I, have been harvested during the present experiment and have been stored at −70° in the culture medium added with 10% dimethyl sulfoxide. The recovery rate after a three-month storage is approximately 30%.

Discussion

As shown in Table I, the range of differentiation inducibility was wider in some T-3-Cl-2 subclones. The possible causative factors are cell growth and the quality of the serum. Although the present data have been obtained by a standardized technique using the same serum, and initiating the experiments with the cells in the same number and in a similar semi-confluent condition, the relatively wider range of benzidine test-positive cell inducibility has been observed on day 5 of the inducer treatment. Most of the T-3-Cl-2 subclones, as listed in Table I, have been passaged over 60 subculture generations, and have become

relatively stable in their differentiation inducibility.

On counting benzidine test-positive cells, some cells are totally covered with blue benzidine crystals, a quinonoid complex, but others merely show focal attachment of the crystals. On day 5 of the treatment with dimethyl sulfoxide, C-10-6 cells with benzidine-positive nuclei increase up to 30~40%, the mechanism of which is still under investigation. Synchronization of the cell growth should be attempted in the future in order to obtain more clarifiable data.

Some of these T-3-Cl-2 subclones have been examined in the reverse transcriptase activity of the culture fluid to know the type C virus situation during erythrodifferentiation, and most of them have shown a peak on day 2 of the inducer treatment. The details were published elsewhere.[6,10]

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References

1) Axelrad, A. A., Steeves, R. A., *Virology*, **24,** 513~518 (1964).
2) Dawson, P. J., Tacke, R. B., Fieldsteel, A. H., *Br. J. Cancer*, **22,** 569~571 (1968).
3) Friend, C., Scher, W., Holland, J. G., Sato, T., *Proc. Natl. Acad. Sci. U.S.A.*, **68,** 378~382 (1971).
4) Furusawa, M., Ikawa, Y., Sugano, H., *Proc. Jpn. Acad.*, **47,** 220~224 (1971).
5) Ikawa, Y., Aida, M., Saito, M., *Gann*, **66,** 583~584 (1975).
6) Ikawa, Y., Inoue, Y., Aida, M., Kameji, R., Shibata, C., Sugano, H., *Bibl. Haematol. (Basel)*, **43,** 37~47 (1976).
7) Ikawa, Y., Ross, J., Leder, P., Gielen, J., Packman, S., Ebert, P., Hayashi, K., Sugano, H., *Proc. 4th Int. Symp. Princess Takamatsu Cancer Res. Fund*, 515~546 (1974).
8) Ikawa, Y., Sugano, H., Furusawa, M., *Bibl. Haematol. (Basel)*, **39,** 955~967 (1973).
9) Ikawa, Y., Sugano, H., Oota, K., *Gann*, **57,** 641~643 (1966).
10) Ikawa, Y., Uchiyama, Y., Aida, M., Obinata, M., *Abstr. Cold Spring Harbor Meet. RNA Tumor Viruses*, 12 (1976).
11) Leder, A., Leder, P., *Cell*, **5,** 319~322 (1975).
12) Obinata, M., Uchiyama, Y., Aida, M., Ikawa, Y., *Abstr. ICN-UCLA Winter Conf. Mol. Mechanisms in the Control of Gene Expression*, 36 (1976).
13) Orkin, S., Harosi, F. I., Leder, P., *Proc. Natl. Acad. Sci. U.S.A.*, **71,** 98~102 (1975).
14) Ostertag, W., Melderis, H., Steinhauder, G., Kluge, N., Dube, S., *Nature (London) New Biol.*, **239,** 231~234 (1972).
15) Patuleia, M., Friend, C., *Cancer Res.*, **27,** 726~730 (1967).
16) Ross, J., Gielen, J., Packman, S., Ikawa, Y., Leder, P., *J. Mol. Biol.*, **87,** 697~714 (1974).
17) Ross, J., Ikawa, Y., Leder, P., *Proc. Natl. Acad. Sci. U.S.A.*, **69,** 3620~3623 (1972).
18) Sassa, S., Takaku, F., Nakao, K., Ikawa, Y., Sugano, H., *Proc. Soc. Exp. Biol. Med.*, **127,** 527~529 (1968).
19) Takahashi, E., Yamada, M., Saito, M., Kuboyama, M., Ogasa, K., *Gann*, **66,** 577~580 (1975).
20) Tanaka, M., Levy, J., Terada, M., Berslow, R., Rifkind, R. M., Marks, P. A., *Proc. Natl. Acad. Sci. U.S.A.*, **72,** 1003~1006 (1975).