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Regulation of Erythroid Differentiation: Characterization of Rauscher and Friend Virus-Transformed Proerythroid Cells (40380)

RAYMOND M. MIAO AND A. HOWARD FIELDSTEEL

Life Sciences Division, SRI International, Menlo Park, California 94025

The elucidation of the mechanism of erythroid differentiation has been greatly facilitated by the availability of Friend virus-transformed erythroid cell lines that can be induced to differentiate and synthesize hemoglobin *in vitro* (1–3). Compounds that induce differentiation include highly polar compounds (1, 4), fatty acids (5), purines and their analogues (6), and certain drugs (7). Cell lines presently available are derived from or are very similar to those originally established by Friend (8, 9). The availability of cell lines with different patterns of response to the various inducers might facilitate the elucidation of the mechanism of induction. In addition, a study of proerythroid cell lines transformed by other leukemia viruses would help to better understand the role of viruses in arresting differentiation. We report some characteristics of two Friend and two Rauscher virus-transformed proerythroid cell lines established in our laboratory that appear to be suitable for comparative studies.

Materials and methods. Chemicals. The following chemicals were used: acetamide, pyridine-*N*-oxide (PNOx), *N*-methyl-2-pyrrolidone (NMP), dimethylformamide (DMF) (Aldrich Chemical Co., Inc., Milwaukee, WI), dimethylsulfoxide (DMSO), *N*-methylformamide (NMF) (J. T. Baker Chemical Co., Phillipsburg, NJ), *n*-butyric acid, hypoxanthine (Sigma Chemical Co., St. Louis, MO), and benzidine dihydrochloride (Mallinckrodt, St. Louis, MO).

Tissue culture. FV-BALB and FV-BDF_1 were established from Friend virus-induced reticulum cell sarcomas in BALB/c and BDF_1 mice, respectively (10, 11). Both of these cell lines have been histologically classified as erythroid precursors (12). RV-133 and RV-187 were established from Rauscher virus-induced reticulum cell sarcomas in BDF_1 mice (12). Cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Flow Laboratories, Inglewood, CA), nonessential amino acids, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cultivation was in a humidified incubator at 37° in an atmosphere of 5% CO_2 in air. The generation times for these cell lines in this medium were approximately 12–14 hr at 37°.

Induction of differentiation. Induction of hemoglobin synthesis was essentially as described by Orkin *et al.* (13). Cells were suspended in culture medium at a concentration of 2×10^3 cells/ml, and 0.1-ml aliquots were added to each well of a 96-well microtiter plate (Costar, Cambridge, MA). Cultures were incubated for 24 hr at 37° at which time 0.1-ml aliquots of the appropriate concentration of inducer, diluted in culture medium, were added to duplicate wells. The cultures were incubated for five more days at 37°. Cells remained spherical but settled on the bottom of the wells. Cells were assayed for hemoglobin by aspirating most of the medium and adding 0.1 ml of phosphate-buffered saline and 0.025 ml of freshly prepared benzidine reagent (0.12% H_2O_2, 0.5% CH_3COOH, and 0.2% benzidine dihydrochloride) to each well. Photographs were taken and the proportion of benzidine-reactive (bz^+) cells were scored out of approximately 200–500 cells counted.

Results. The dose response of virus-transformed RV-133 to the different inducers, none of which were toxic at the concentrations used here, is shown in Fig. 1. Benzidine-reactive cells (bz^+) were induced with all of the compounds tested. The spontaneous level of differentiation was 5% bz^+. The highest levels of induction occurred with 10 mM NMP (59% bz^+) and 140 m*M* DMSO (58% bz^+). Butyric acid at 1 m*M* exhibited the lowest concentration required for induction of differentiation; however, the maximum level of differentiation with butyric acid was

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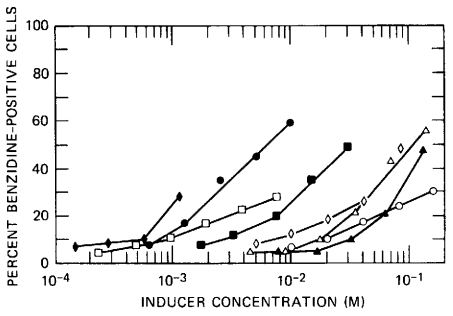

FIG. 1. Dose response of RV-133 cells to various inducers. RV-133 cells were grown in the presence of the indicated concentrations of inducers for 5 days at 37°. Cultures were then assayed for hemoglobin by the benzidine test. The percent of benzidine-responsive (benzidine-positive) cells was then calculated out of about 200–500 cells counted. The inducers were butyric acid (◆), N-methylpyrrolidone (●), hypoxanthine (□), pyridine-N-oxide (■), N-methylformamide (◇), dimethylsulfoxide (△), dimethylformamide (▲), and acetamide (○).

only 28% bz^+. The absolute number of bz^+ cells did not increase at concentrations of inducers higher than those shown in Fig. 1. Cultivation in the presence of inducers for more than 5 days did not result in a higher percentage of bz^+ cells. The level of induction of RV-133 was compared to that of C1 745, the prototype Friend virus (FV)-transformed proerythroid cell line (data not shown). The spontaneous level of bz^+ cells was the same (5%) for both RV-133 and C1 745. Cells were exposed for 5 days to the highest concentration of each inducer shown in Fig. 1. All but two of the compounds tested (NMF, butyric acid) induced a much higher percentage of bz^+ cells with C1 745 than with RV-133.

As shown in Fig. 2, Rauscher virus (RV)-transformed proerythroid cell line, RV-187, also responded to the eight inducers. The spontaneous level of bz^+ cells was 2%. The maximum levels of induction are lower than with RV-133 except in the presence of PNOx, which induced a maximum of 60% bz^+ cells with RV-187 compared to 50% bz^+ cells with RV-133. Concentrations of inducers higher than those shown in Fig. 2 were cytotoxic for RV-187. The maximum noncytotoxic concentrations of inducers was at least one-half the concentrations for RV-133 with the exception of DMSO and NMF. Both RV-133 and RV-187 cells were, therefore, induced to differentiate in the presence of all eight compounds tested, although the level of differentiation and dose-response were different for the two cell lines and the various inducers.

The effects of the same inducers on two FV-transformed proerythroid cell lines, FV-BALB and FV-BDF_1 are shown in Table I. The FV-BALB cell line had an extremely low level of spontaneous differentiation. The estimated level was <0.05% or zero bz^+ cells out of about 2000 cells. The data show that with seven inducers over the concentration range previously shown to be effective with RV-133, RV-187, and C1 745, there was no

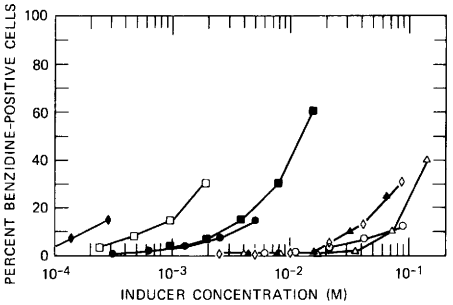

FIG. 2. Dose response of RV-187 cells to various inducers. Cells were treated as described in the legend to Fig. 1.

TABLE I. LACK OF INDUCTION OF HEMOGLOBIN IN FV-BALB AND FV-BDF_1 CELLS.

Inducer	Concentration (mM)	Benzidine-positive cells (%)[a] FV-BALB	FV-BDF_1
	—		
None	—	<0.05	1
Dimethylsulfoxide	8.75–280	<0.05	1
Dimethylformamide	8.15–260	<0.05	1
N-methylformamide	10.5–334	<0.05	1
Pyridine-N-oxide	3.75–120	<0.05	1
Butyric acid	0.071–2.28	<0.05	1
Hypoxanthine	0.47–15	<0.05	1
Acetamide	10.5–334	<0.05	1
N-methylpyrrolidone	40	toxic	1
	20	23	1
	10	7	1
	1.25–5	<0.05	1

[a] Cells were exposed to inducers in the concentration ranges shown. After five days, the cells were treated with benzidine reagent and the percent of benzidine-reactive cells was determined.

response with the FV-BALB cell line. The concentration range of each inducer used included cytotoxic concentrations. Only exposure to 20 m*M* NMP induced hemoglobin synthesis in these cells. The second FV-transformed cell line, FV-BDF_1, had a higher level of spontaneous differentiation (1% bz^+ cells) than did FV-BALB, but it was lower than that for either of the RV-transformed cell lines or for Cl 745. In contrast to all of the other cell lines tested none of the eight compounds tested induced hemoglobin synthesis in FV-BDF_1. Again, the concentrations of compounds tested included cytotoxic levels.

Discussion. The data presented here show that the RV-transformed proerythroid cell lines, RV-133 and RV-187, established in this laboratory are similar to the FV-transformed proerythroid cell line Cl 745 in that they can be induced to differentiate along the erythroid line. RV-133 and RV-187 are, however, clearly different from Cl 745 in their dose-response to the inducers tested and in the fact that they were transformed by and continue to produce Rauscher virus (12). We have not yet been able to demonstrate as large a percent differentiation in RV-133 or RV-187 cultures as occurs with Cl 745 (60% vs 100% bz^+) which may merely reflect heterogeneity in the RV-transformed cultures. Variation in inducibility has been shown to exist in subclones of Cl 745 (14). Since neither of the RV-transformed cell lines have been cloned, it may be possible to isolate a clone from them that exhibits a dose-response pattern similar to that for Cl 745.

It is possible that RV-133 and RV-187 represent erythroid precursor target cells that are different from Cl 745 cells. It is not known whether the two viruses share the same target cells *in vivo* (15–17). Several lines of evidence indicate that the Friend virus target cell is a late-committed erythroid stem cell (18, 19) whereas Rauscher virus may be capable of infecting uncommitted stem cells also (17, 20). Evidence suggesting that RV-133 and RV-187 may be physiologically different from Cl 745 has recently been reported (21). The potent tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate was shown to induce hemoglobin synthesis in both RV-transformed cell lines but to inhibit both spontaneous and DMSO-induced hemoglobin synthesis in Cl 745. In addition, we have recently found that vitamin A and other retinoids also have completely opposite effects on hemoglobin synthesis in RV-133 and Cl 745 cells (manuscript in preparation).

Previous studies were centered on Friend virus and Friend virus-transformed cells since no other virus-transformed proerythroid cell lines had been established. The cell lines, RV-133 and RV-187, are the first known RV-transformed proerythroid cell lines that can be induced to differentiate. Recently, de Both *et al.* (22) reported the establishment of an RV-transformed proerythroid cell line from DBA mice. The interesting aspect of those cells is that they synthesized hemoglobin upon exposure to erythropoietin (Epo) alone. FV-transformed proerythroid cells do not respond to Epo alone. The response of our cell lines to Epo has not yet been determined.

The FV-BDF_1 and FV-BALB cell lines are clearly different from the other cell lines. Unlike Cl 745, FV-BDF_1 does not produce any infectious Friend virus (11) but does produce noninfectious C-type particles (our unpublished data). Moreover, we have now shown that it is not induced to differentiate by exposure to any of eight known inducers of Cl 745. Other investigators have shown that subclones of highly inducible FV-transformed proerythroid cell lines can be isolated that show decreased levels of induction (bz^+) in the presence of DMSO (14, 23, 24). It is not known whether the virus or the cell is responsible for the lack of induction of these cells. However, since it appears that induction of differentiation is not dependent on complete viral gene expression (24, 25), noninducibility may be due to the physiological state of these cells. Studies are continuing to find a compound or combination of compounds that will induce some degree of differentiation in FV-BDF_1 cultures.

The FV-BALB cell line is similar to FV-BDF_1 in that it does not produce infectious Friend virus but does contain the retrievable Friend virus genome (26). In contrast to FV-BDF_1 it does not produce any C-type particles (27). We have now shown that, like FV-BDF_1, it does not synthesize hemoglobin upon exposure to several known inducers of Cl 745. However, unlike FV-BDF_1, it can be induced to differentiate to a small degree by

exposure to NMP. This suggests that NMP may have a mechanism of action different from that of the other compounds tested and also shows that FV-BALB are in fact proerythroid cells.

It is hoped that the characteristics of the FV- and RV-transformed proerythroid cells described here will make them useful in future studies on the molecular and genetic regulation of gene expression.

Summary. The induction of differentiation in two Friend and two Rauscher virus-transformed proerythroid cell lines was studied. The benzidine assay for hemoglobin was used to determine differentiation. The Rauscher virus-transformed lines, RV-133 and RV-187, were induced to differentiate in the presence of eight known inducers, including dimethyl-sulfoxide, butyric acid, and *N*-methylpyrrolidone. One Friend virus-transformed line, FV-BDF_1 did not differentiate on exposure to any inducer. The second Friend virus-transformed line, FV-BALB, differentiated only on exposure to *N*-methylpyrrolidone.

1. Friend, C., Scher, W., Holland, J. G., and Sato, L., Proc. Nat. Acad. Sci. **68,** 378 (1971).
2. Ikawa, Y., Furusawa, M., and Sugano, H., Bibl. Haematol. **39,** 955 (1973).
3. Ostertag, W., Melderis, H., Steinheider, G., Kluge, N., and Dube, S., Nature New Biol. **239,** 231 (1972).
4. Tanaka, M., Levy, J., Terada, M., Breslow, R., Rifkind, R. A., and Marks, P., Proc. Nat. Acad. Sci. **72,** 1003 (1975).
5. Leder, A., and Leder, P., Cell **5,** 319 (1975).
6. Gusella, J. E., and Housman, D., Cell **8,** 263 (1976).
7. Burnstein, A., Hunt, D. B., Crichley, V., and Mak, T. W., Cell **9,** 375 (1976).
8. Scher, W., Holland, J. G., and Friend, C., Blood **37,** 428 (1971).
9. Scher, W., Preisler, H. D., and Friend, C., J. Cell. Physiol. **81,** 63 (1973).
10. Fieldsteel, A. H., Dawson, P. J., and Scholler, J., J. Nat. Cancer Inst. **36,** 71 (1966).
11. Fieldsteel, A. H., Kurahara, C., and Dawson, P. J., Cancer Res. **29,** 1846 (1969).
12. Fieldsteel, A. H., Dawson, P. J., Becker, F. A., Kurahara, C., and Mitoma, C., Brit. J. Cancer **35,** 199 (1977).
13. Orkin, S. H., Harosi, F. I., and Leder, P., Proc. Nat. Acad. Sci. **72,** 98 (1975).
14. Rovera, G., and Bonaiuto, J., Cancer Res. **36,** 4057 (1976).
15. Friend, C., Patuleia, M. C., and de Harven, E., NCI Monogr. **22,** 505 (1966).
16. Pluznick, D. H., Sachs, L., and Resnitsky, R., NCI Monogr. **22,** 2 (1966).
17. Brommer, E. J. P., and Bentvelzen, P., Europ. J. Cancer **10,** 827 (1974).
18. Harrison, P. R., Nature (London) **262,** 353 (1976).
19. Wallace, R. B., Dube, S. K., and Bonner, J., Science **198,** 1166 (1977).
20. OKunewick, J. P., and Chervenick, P. A., Proc. Soc. Exp. Biol. Med. **155,** 382 (1977).
21. Miao, R. M., Fieldsteel, A. H., and Fodge, D. W., Nature, (London) **274,** 271 (1978).
22. De Both, N. J., Vermey, M., Van't Hull, E., Klootwijk-Van-Dijke, Van Griensven, L. J. L. D., Mol, J. N. M., and Stoof, T. J., Nature (London) **272,** 626 (1978).
23. Paul, H., and Hickey, I., Exp. Cell Res. **87,** 20 (1974).
24. Ikawa, Y., Inoue, Y., Aida, M., Kameji, R., Shibata, C., and Sugano, H., Bibl. Haematol. **43,** 37 (1976).
25. Swetley, P., and Ostertag, W., Nature (London) **251,** 642 (1974).
26. Fieldsteel, A. H., Dawson, P. J., and Kurahara, C., Int. J. Cancer **8,** 304 (1971).
27. Black, D. R., and Fieldsteel, A. H., Int. J. Cancer **17,** 245 (1976).

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