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SULFHYDRYL GROUPS AND THE DIFFERENTIATION OF MURINE ERYTHROLEUKEMIA CELLS

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SUMMARY

The importance of cysteine and sulfhydryl groups has been demonstrated in relation to the differentiation and respiration of Friend erythroleukemia cells (FLC). The respiratory rate of undifferentiated FLC was higher basally (5.06 ± 0.16 vs. 3.10 ± 0.09 nmoles O_2/min/10^6 cells) and was further 70% stimulated by addition of cysteine, whereas DMSO-induced differentiated cells were insensitive. A sulfhydryl blocking agent (PCMS) was capable of maintaining the differentiated state of FLC cultured in the absence of DMSO and this effect appeared to be reversible upon removal of the PCMS.

INTRODUCTION

Although it has been previously hypothesized that the intracellular level of sulfhydryl groups may play an important role in the metabolism of malignant mammalian cells (1) and the mechanism of action of some potent antileukemic drugs (benzofuranzane and α methylene lactones) can be related to their reaction with key intracellular thiol groups (2-4), there has been very little work on the role of sulfhydryl groups on tumor cell differentiation. Since Friend leukemia cells (FLC) exist in vitro in both undifferentiated and chemically-induced differentiated forms and the transition and manifestations of the differentiation process have been carefully characterized (5-7), we have used this model to study the possible role of sulfhydryl groups in erythroleukemia cell differentiation and respiration.

MATERIALS AND METHODS

FLC were plated at 10^5 cells/ml and cultured in Dulbecco's modified Eagle's medium containing 15% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 95% air, 5% CO_2 atmosphere at 37 ± 3.5° C. Hemoglobin concentrations were measured using the technique of Crosby and Furth (8).

In experiments designed to evaluate the effect of cysteine on the respiration of both differentiated and undifferentiated FLC, respiration was measured with a Clark-type oxygen electrode (Yellow Spring Inst., Ohio) as previously described (9). The reaction was started by pipetting 200 µl of a well-aerated cell suspension

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into a 37° C reaction chamber containing 1.3 ml of respiratory buffer (50mM dimethylglutaric acid, pH 7.1, 1% glucose, 1mM $CaCl_2$) (10) saturated with oxygen (217 nmoles O_2/ml). Before measurements, the cells were centrifuged, and resuspended in the respiratory buffer (37°C). During measurements, the suspensions were continuously mixed by a magnetic stirring bar. Results were expressed as nmoles O_2/min/10^6 cells. No chemical oxidation of cysteine occurred in the cell free respiratory buffer (excluding the possibility of artifact).

RESULTS

The sensitivity of cytochrome-mediated electron transport to potassium cyanide (KCN), which inhibits the reduction of O_2 by cytochrome a in the respiratory chain, was studied in both differentiated and undifferentiated FLC. As shown in Figure 1A, both the control and the DMSO-treated cells had a similar sensitivity to KCN; half maximal inhibition in control cells was noted at 129μM KCN whereas in DMSO-treated cells, it was noted at 118μM KCN. These data suggested that the cytochrome pathway was at least qualitatively similar for both undifferentiated and differentiated cells.

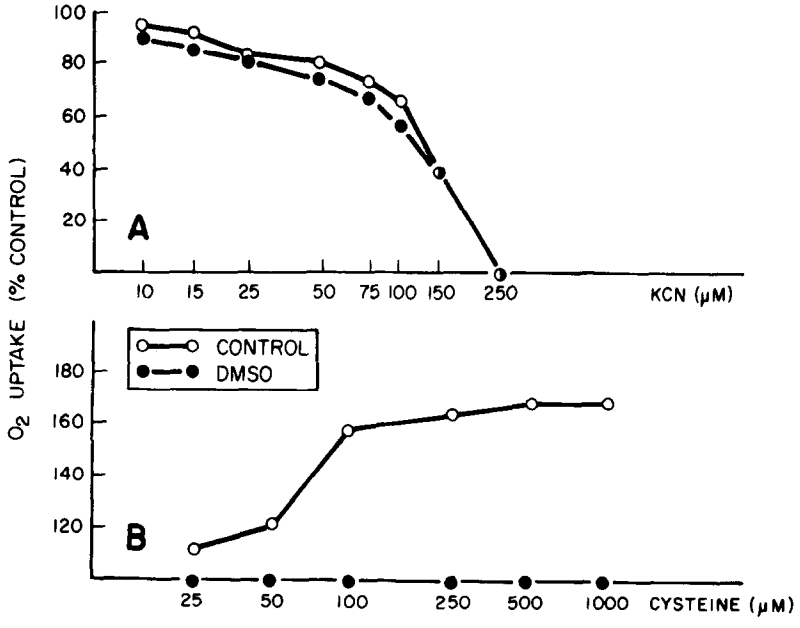

Figure 1. **1.0 x 10^5 undifferentiated FLC/ml were plated in culture medium with or without 1.5% DMSO. After 5 days, both control and DMSO-treated cells were washed and resuspended in the respiratory buffer. o———o, control; •-----•, DMSO.**

Since cysteine has been previously shown to stimulate respiration only of the non-differentiated form of the dimorphic fungus, histoplasma capsulatum (13), we evaluated the effect of this amino acid on the respiration of both differentiated and undifferentiated FLC. Figure 1B shows the effect of different concentrations of cysteine on cellular respiration by FLC. The basal respiration rate was signifiantly higher in the undifferentiated than in DMSO-treated FLC (undifferentiated or non-DMSO-treated, - 5.06 ± 0.16; DMSO - differentiated, 3.10 ± 0.09 nmoles O_2/min/10^6 cells; $p < 0.001$). While respiration of DMSO-treated cells (5 x 10^6 cells/ml) was completely insensitive to the addition of exogenous cysteine, the respiration of the same number of undifferentiated FLC was stimulated up to 70% over the basal respiratory rate. This cysteine-stimulated respiration was prevented by treatment of the cells with 250μM KCN, demonstrating that this respiration was mediated by the cytochrome system. This respiratory stimulation by cysteine was accomplished despite a lower uptake of ^{35}S-labelled cysteine (undifferentiated FLC-17.16 ± 0.90; DMSO-treated FLC - 32.24 ± 2.90 moles/min/10^6 cells), suggesting that cysteine uptake is not rate-limiting.

In order to evaluate whether the change in cysteine-stimulated respiration is related to the differentation process in FLC (as it is in other eukaryotic systems), we have studied the effect of PCMS (p-chloromercuriphenylsulfonic acid, a sulfhydryl blocking agent) on hemoglobin concentration as a manifestation of FLC differentation.

After five days of treatment with 1.5% dimethylsulfoxide (DMSO), more than 90% of the FLC contained hemoglobin as detected by the benzidine test. After a 6-day period, DMSO treated cells (1.6 x 10^6 cells/ml) produced 9.04μg hemoglobin (Hb)/10^6 cells. Cultures were then divided into three treatment groups: control (no DMSO), DMSO, and 5μM PCMS, and replated at 10^5 cells/ml under identical conditions. In control experiments it was found that treatment of undifferentiated FLC (10^5 cells/ml) with PCMS did not produce any changes in either the rate of replication (control - 4.33 ± 0.19; PCMS - 3.79 ± 1.07 x 10^6 cells/ml; $p > 0.05$) or in hemoglobin production (control - 0.73 ± 0.03; PCMS - 0.81 ± 0.01μg Hb/10^6 cells; $p > 0.05$).

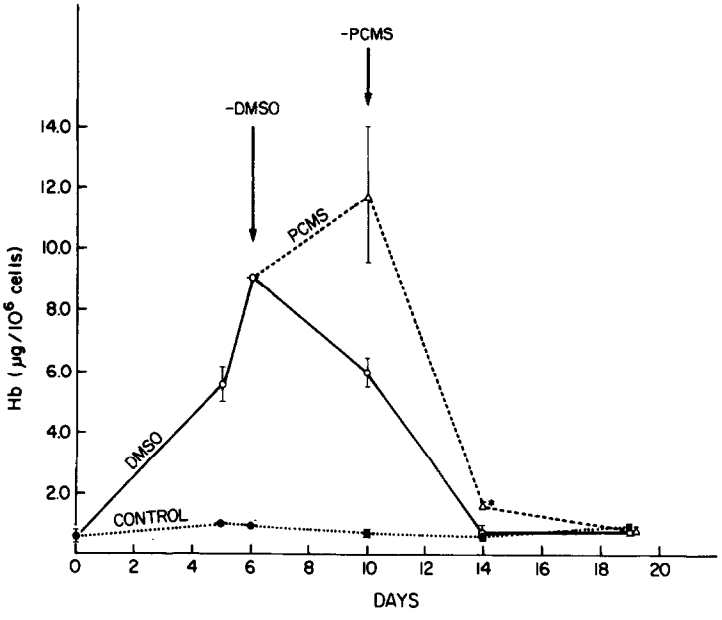

Figure 2. 1.0 x 10^5 undifferentiated FLC/ml were plated in 75 cm^2 plastic T flasks containing 10 ml of culture medium with or without 1.5% DMSO. Six days later, cells were washed and resuspended in DMSO - free medium with or without 5 µM PCMS. Four days after this last plating, PCMS-treated cells, as well as control (no DMSO) and DMSO-control cells were washed and plated in culture medium (at 1.0 x 10^5 FLC/ml) without either DMSO or PCMS. Hemoglobin was measured from cell lysates using the technique of Crosby and Furth (8). Data are presented as mean ± S.E.M. of replicate analyses of duplicate cultures * = $p < 0.05$. •....•, control; o———o, DMSO; Δ----Δ, PCMS.

Removal of the DMSO from the culture medium of differentiated FLC resulted in the expected decrease in hemoglobin production and, after 8 days (7.8 duplication cycles), the hemoglobin levels were identical to those of undifferentiated control cells (0.59 ± 0.01µg Hb/10^6 cells) (Figure 2). In the absence of DMSO, FLC also gradually returned to their initial rapid doubling time (after 4 days - 40.0 hours; after 8 days - 17.8 hours). After removal of the DMSO and washing the cells, treatment of the DMSO-differentiated cells for 4 additional days with PCMS not only prevented the cells from returning to the initial doubling time (Table 1), but also increased the FLC

TABLE 1. VIABLE CELL COUNTS AND HEMOGLOBIN CONCENTRATIONS

	96 Hours			192 Hours			312 Hours	
	Number of viable cells x 10^6/ml	Hemoglobin µg/10^6 cells		Number of viable cells x 10^6/ml	****Hemoglobin µg/10^6 cells		Number of viable cells x 10^6/ml	Hemoglobin µg/10^6 cells
Control*	4.33 ± 0.19 (5.4)**	0.73 ± 0.03	Control	5.38 ± 0.16 (5.7)	0.66 ± 0.07	Control	5.62 ± 0.02 (5.8)	1.11 ± 0.03
- DMSO	0.54 ± 0.05 (2.4)	5.98 ± 0.60	- DMSO	4.17 ± 0.01 (5.4)	0.59 ± 0.004	- DMSO	5.50 ± 0.00 (5.8)	0.93 ± 0.08
+ PCMS	0.19 ± 0.02 (0.9)	11.69 ± 2.36	- PCMS	1.65 ± 0.06 (4.1)	1.65 ± 0.13	- PCMS	5.29 ± 0.25 (5.8)	0.93 ± 0.06
+ DMSO	0.19 ± 0.02 (0.9)	12.04 ± 2.87	+ DMSO	0.11 ± 0.01 (0.1)	11.34 ± 2.46	+ DMSO***	0.08 ± 0.01 (<0.1)	9.92 ± 1.08

* Control cells refer to undifferentiated (non DMSO-treated) cells

** Number in parentheses refers to the number of duplication cycles

*** By 312 hours of DMSO-treatment, cell viability was only 91%

**** Cell viability was determined by trypan blue exclusion and exceeded 97% except where specifically noted by***

hemoglobin content to 11.69 ± 2.36μg Hb/10^6 cells. This effect was identical to that observed when the differentiated FLC were replated for 4 days with DMSO (12.04 ± 2.87μg Hb/10^6 cells). Similarly, both PCMS and DMSO had identical effects on the mean doubling time of differentiated cells (108 hours). Removal of the PCMS from the medium after the 4-day period produced the same apparent effects as those noted after removal of DMSO and by 9 days (3.9 duplication cycles) the hemoglobin contents and doubling times were identical to those of undifferentiated FLC (11).

The numbers of viable cells and concentrations of hemoglobin under these experimental conditions are compiled in Table 1. Ninety-six hours after removal of DMSO and replating (at 10^5 cells/ml) in control medium, the hemoglobin concentration had fallen to 5.98 ± 0.60μg/10^6 cells, but there was still a significant inhibition of proliferation noted (0.54 ± 0.05 vs control 4.33 ± 0.19 x 10^6 cells/ml and 2.4 vs control 5.4 duplication cycles). These data are consistent with previous suggestions that the return to the undifferentiated state is due to replication of the few residual undifferentiated or non-terminally differentiated cells (12) which retain the capacity for cell division. However, the data for PCMS-treated cells are more interesting. Within 96 hours after removal of PCMS and replating in control medium (at 10^5 cells/ml), the hemoglobin concentration had fallen from 11.69 ± 2.36 to 1.65 ± 0.13μg/10^6 cells. However, this rapid change in cell hemoglobin content occurred with only a relatively small alteration in the replication rate (1.65 ± 0.06 vs control 5.38 ± 0.16 x 10^6 cells/ml and 4.1 vs control 5.7 duplication cycles). Since the replated cultures contained no more than 10^4 undifferentiated cells/ml (10% of the total population), 5.7 duplication cycles would result in no more than 0.54 x 10^6 undifferentiated cells/ml (as was observed in the four day period after removal of DMSO-see Table 1). However, as already stated, after removal of the PCMS, the cultures contained 1.65 x 10^6 cells/ml. There are two possible interpretations. If DMSO - maintained differentiation were irreversible, the high content of differentiated cells (>65% or at least 1.11 out of 1.65 x 10^6 viable cells/ml) should have resulted in a high hemoglobin content. Since this was not observed and the number of duplication cycles (at least 3.6 to yield 1.11 x 10^6 cells/ml) required rapid replication,

the data are more consistent with the possible explanation that PCMS - maintained differentiation is reversible. In order to eliminate the possibility that PCMS alone affects the replication of undifferentiated cells at lower cell densities, FLC were plated at 10^3 , 5×10^3 , 10^4 , and 5×10^4 cells/ml and cultured for 4 days in the presence and absence of 5 μM PCMS. PCMS did not alter rates of cell replication at any cell density tested (10^3 - PCMS 0.16, control 0.17 x 10^5 cells/ml; 5×10^3 - PCMS 0.82, control 0.77 x 10^5 cells/ml; 10^4 - PCMS 1.97, control 1.78 x 10^5 cells/ml; 5×10^4 - PCMS 0.82, control 0.76 x 10^6 cells/ml). Thus, the data are most consistent with the concept that PCMS - maintained differentiation of FLC may be reversible.

DISCUSSION

These studies have resulted in two new findings, that cysteine stimulates oxygen consumption in undifferentiated but not in DMSO-differentiated FLC, and that PCMS, a sulfhydryl blocker, stimulates chemically-induced differentiated FLC to continue to synthesize hemoglobin after removal of DMSO, whereas it has no effect on undifferentiated cells.

These observations on Friend leukemia cells are similar to those we have recently described in a lower eukaryotic system, Histoplasma capsulatum (13). In this system, the unicellular, pathogenic, non-differentiated yeast phase requires cysteine for its expression, while the multicellular saprobic, differentiated mycelial phase does not; sulfhydryl blocking agents prevent the transition from the differentiated form to the undifferentiated yeast phase; finally, cysteine stimulates cellular respiration only in the yeast phase, an effect which is detectable very shortly after the initiation of the transition from mycelium to yeast. In this effect on stimulation of respiration, cysteine does not act as an electron donor, rather it directly activates mitochondria resulting in oxidation of endogenous substrates; this effect has also been observed in experiments using β-mercaptoethanol, and dithiothriotal, suggesting that the sulfhydryl groups are critical (unpublished observations). Similar effects of cysteine on stimulation of respiration have also been observed during germination of bacteria (14), during conversion of spores to hyphae in Neuropora crassa (15), and after fertilization in sea urchin eggs (16).

Although it is clear that differentiation of Histoplasma capsulatum is reversible, previous studies have suggested that DMSO-induced differentiation of FLC is essentially irreversible and that return of the cultures to total dedifferentiation occurs only after overgrowth of residual DMSO-resistent cells (7). Our data are consistent with this observation. We have previously demonstrated that in the presence of DMSO plus a long-acting synthetic analogue of prostaglandin E_2, differentiation of Friend leukemia cells can occur without cell replication (11). The current studies have suggested that in contrast to the observations for DMSO, differentiation maintained by PCMS may be reversible and not dependent only upon replication of undifferentiated FLC. This implies that PCMS may induce a biochemical alteration in differentiated FLC, presumably related to the oxidation state of some critical sulfhydryl groups.

In summary, the data substantiate the importance of the oxidation state and/or the level of sulfhydryl groups in the expression of tumor cell differentiation. This similarity with Histoplasma opens new vistas for the study of the differentiation process in malignant mammalian cells.

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