

# Mode of Differentiation of Human Promyelocytic Leukemia Cell Line, HL-60, by $1\alpha,25$ -Dihydroxyvitamin $D_3$

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**ABSTRACT.** Differentiation of human promyelocytic leukemia cells, HL-60, has been extensively studied. In this study we utilized  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $D_3$ ) as a potent inducer and cellular superoxide production as the functional differentiation marker. We examined how nitroblue tetrazolium (NBT) positive cells were produced in the presence of  $D_3$ . Growth of HL-60 cells tended to level off when the curve was drawn on a logarithmic scale but they grew linearly on a normal scale. When absolute numbers of NBT positive or negative cells were plotted, NBT positive cells only increased linearly in a normal scale, whereas NBT negative cells remained constant after they doubled themselves. When cells were sparsely inoculated in 0.3% agar and cultured for 4 days in the presence of  $D_3$ , clusters of cells were stained with NBT. Each cluster of cells was composed of one or two NBT negative and three to six NBT positive cells. After treatment with nocodazole and  $D_3$ , cells were cultured further in the presence of  $D_3$ . It was demonstrated that only NBT positive cells increased abruptly. Based on these results, differentiated cells might be produced and accumulate through mitosis. In the presence of  $D_3$  NBT negative cells remained constant in number and continued to produce NBT positive cells, working as so-called stem cells.

Keyword: cell differentiation, cholecalciferols

## INTRODUCTION

The human leukemic cell line (HL-60), derived from a patient with acute promyelocytic leukemia, has been maintained in continuous culture (1). These promyelocytes can be induced to differentiate into granulocytes (2-4), macrophages or monocytes (5,6), eosinophils (7) and basophils (8) by various chemical inducers and culture conditions. Among various agents  $1\alpha,25$ -dihydroxyvitamin  $D_3$  and retinoic acid are very important clinically. Exposure to  $D_3$  has been found to result in terminal monocytic differentiation, morphologically and functionally (9, 10).

In the presence of  $D_3$  approximately 80% of

HL-60 cells became nitroblue tetrazolium (NBT) positive, but the rest remained negative (9-11). There are several reasons for the presence of NBT negative cells: 1) the amount of  $D_3$  was insufficient; 2) the treatment period was short; 3) HL-60 cells used in these previous experiments were heterogeneous; and 4) a certain population of HL-60 cells might work as so-called stem cells to produce NBT positive cells. In this study we used a potent inducer,  $1\alpha,25$ -dihydroxyvitamin  $D_3$  for monocytic differentiation. Cell differentiation was assayed by the occurrence of the functional differentiation marker, inducible cellular superoxide production. We examined these possibilities by cell kinetic analysis and microscopically.

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## MATERIALS AND METHODS

### Culture cells

HL-60 cells were supplied by these Japanese Cancer Research Resources Bank, Tokyo. Cells were cultured in RPMI 1640 (Gibco), supplemented with penicillin (100 u/ml), streptomycin (100 µg/ml) and 10% heat-inactivated fetal bovine serum (Cell Culture Laboratories, Ohio). Unless stated, cells were inoculated at a concentration of  $2 \times 10^4$  cells/ml in 5 ml of medium. Cells were grown at 37 C in an atmosphere of 5% CO<sub>2</sub>. For the assay of cell population cells were suspended in 0.25% trypan blue in phosphate buffered saline (NaCl 8.0 g/l, KCl 0.2 g/l, Na<sub>2</sub>HPO<sub>4</sub> 1.15 g/l, KH<sub>2</sub>PO<sub>4</sub> 0.2 g/l) and counted with the aid of a hemocytometer at indicated times.

### Assay for differentiation

Myeloid differentiation was assayed by the phorbol myristate acetate (PMA) (Sigma Chemical) induced superoxide production, which has been considered as a hallmark for mature myeloid cells. The method was described previously (13,14). At least 300 cells were scored for each determination using a hemocytometer.

### Chemicals

Nitroblue tetrazolium and 1 $\alpha$ ,25-dihydroxy-vitamin D<sub>3</sub> (lot number ECL2791) were purchased from Wako Pure Chemical Industries, Ltd. Other chemicals were of analytical grade. A potent inducer, D<sub>3</sub>, was first dissolved in dimethyl sulfoxide at a concentration of 2.5 mM and diluted 100 fold with ethanol to a concentration of 25 µM. This diluted D<sub>3</sub> was added to the culture medium at a ratio 1 µl of diluted D<sub>3</sub> to 1 ml of culture medium. Final concentrations of dimethyl sulfoxide and ethanol were less than 0.001% and 0.1%, respectively. At these concentrations of vehicles

no biological effect on cell growth or on cell differentiation rate was detected. Unless stated, the concentration of D<sub>3</sub> was 25 nM.

## RESULTS

### Concentration of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>

In previous reports, the concentrations of D<sub>3</sub> varied from 2 to 120 nM in differentiation experiments (10,14,15). As a preliminary experiment, therefore, we determined the effective concentration of D<sub>3</sub> in our system. HL-60 cells were inoculated at  $2 \times 10^4$  cells/ml and cultured for 4 days in the presence of various concentrations of D<sub>3</sub>. Cell numbers and differentiation rates were determined as described in MATERIALS AND METHODS. The rate of NBT positive cells started to increase at  $1 \times 10^{-11}$  M of D<sub>3</sub> and reached maximum at the concentration of about  $5 \times 10^{-8}$  M. At concentrations higher than  $5 \times 10^{-8}$  M the percentage of NBT positive cells leveled off. Effect of D<sub>3</sub> on cell growth was also determined. It was found that cell growth was not affected until the concentration of  $5 \times 10^{-8}$  M of D<sub>3</sub>. At concentrations higher than  $5 \times 10^{-8}$  M cell debris was detected in the culture medium and cell number started to decrease. In the experiments thereafter, D<sub>3</sub> was used at the concentration of 25 nM.

### Cell growth

Effect of D<sub>3</sub> on cell growth was examined as a function of time. HL-60 cells in logarithmic phase were inoculated at a concentration of  $2 \times 10^4$  cells/ml in 55 mm petri dishes. Cell number was determined at the indicated times (Fig. 1). In control series, which received only the vehicle, cells grew linearly in a logarithmic scale, whereas cells which were treated with D<sub>3</sub> showed less growth than the control cells. The cell growth in D<sub>3</sub> treated culture, however, increased steadily during the experimental period.

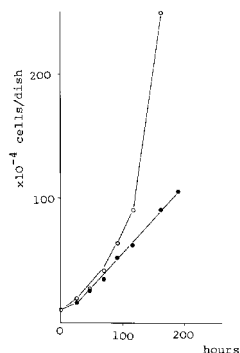


Fig. 1. Cell growth in the presence (●) or absence (○) of  $D_3$  (25 nM). Cells were plated in 5 ml medium/dish at a concentration of  $2 \times 10^4$  cells/ml. Cell numbers were determined at indicated times. The abscissa is time in hour and the ordinate cell number/dish. Cell growth was plotted in a normal scale.

When these growth curves were drawn on a normal scale, the curve of  $D_3$  treated cells became linear (Fig. 1), which suggested that in the presence of  $D_3$  a certain portion of HL-60 cells might become nondividing cells. On the other hand, control cells showed exponential growth.

### Differentiation assay

It has been documented that HL-60 cells differentiate into monocytes or macrophages in the presence of  $D_3$  (9-11). Cell differentiation was assayed by inducible cellular superoxide production as the functional differentiation marker. NBT positive cells were determined as described in MATERIALS AND METHODS. In control series this cell line produced 3-4% of NBT positive cells spontaneously. On the other hand, the ratio of NBT positive to total cells began to increase after one generation time (40 hours) in the presence of  $D_3$ . The first part of the increasing curve was concave, but after then the increment was linear and the ratio leveled off after 120 hours.

The percentage of NBT positive to total cells increased linearly from the 40th to the 120th hour (data not shown) and in Fig. 1 cells grew linearly except for the first 40 hours in a normal scale in the presence of  $D_3$ . These two

results prompted us to calculate the absolute numbers of NBT positive and negative cells, respectively. Fig. 2 demonstrated that only NBT positive cells grew linearly but NBT negative cells remained constant after doubling themselves in the presence of  $D_3$ . The curve of NBT positive cells was similar to that of total cell growth as shown in Fig. 1 drawn in a normal scale. Namely, it could be concluded that increasing cells in Fig. 1. were NBT positive ones.

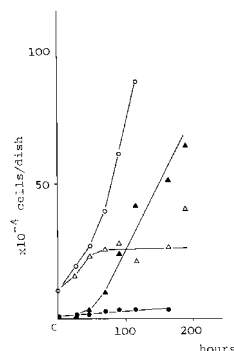


Fig. 2. Effects of  $D_3$  on NBT positive and negative cells. Cell number and the percentage of NBT positive cells were determined at indicated times. The numbers of NBT positive and negative cells were calculated from these data. The abscissa is time in hour and the ordinate cell number/dish. Control NBT positive (●) and negative (○) and  $D_3$  treated cells (NBT positive (▲) and negative (△)).

In this study we used HL-60 cells obtained from the Japanese Cancer Research Resources Bank. This cell line might be heterogeneous. To exclude the possibility that there were at least two cell lines: one proliferative cells became NBT positive and the other nonproliferative cells remained NBT negative in the presence of  $D_3$ , nine cell lines were cloned from the original HL-60 cells by limiting dilution. Table 1 shows that all the cloned cells became NBT positive in the presence of  $D_3$ , ranging from 40 to 70%. This experiment demonstrated that the original HL-60 cells were almost homogeneous in terms of capability of differentiation in the presence of  $D_3$ , although the rates of differentiation were diverse.

Table 1. The percentage of NBT positive cells in nine clones and original cells

clones	NBT positive cells (%) <sup>a</sup>	
	control	D <sub>3</sub>
A	4.59	70.3
C	6.45	70.4
D	3.92	63.3
E	7.71	72.5
F	1.83	38.0
G	2.19	50.9
H	3.67	52.7
N	7.11	52.5
M	8.54	64.4
Original	2.84	50.3

<sup>a</sup>Original and nine clones were cultured in the presence or absence of D<sub>3</sub> for 4 days. NBT positive cells were counted and their percentages calculated.

### Removal of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>

In the next experiment HL-60 cells which had been treated with D<sub>3</sub> for 4 days were placed in inducer-free medium and cell number was reduced to 2 x 10<sup>4</sup> cells/ml. Cell growth and absolute numbers of NBT positive and negative cells were examined. The cells started to grow exponentially about 50 hours after placement into inducer free medium, indicating that D<sub>3</sub> treated cells were reversible in growth capability. As shown in Fig. 3a, after replacement with D<sub>3</sub> free medium the absolute number of NBT negative cells started growing exponentially just like total cell growth, whereas NBT positive cells remained constant during the experiment. On the other hand, D<sub>3</sub> treated cells were placed in a new culture medium containing D<sub>3</sub> at the same concentration. Cell growth was linear from the beginning of culture in a normal scale. It was determined that NBT positive cells increased linearly in a normal scale but NBT negative cells remained constant (Fig. 3b).

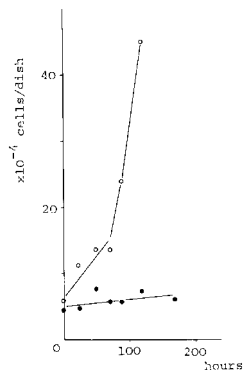


Fig. 3. a) Time course of NBT positive or negative cells. Cells had been cultured in the presence of D<sub>3</sub> for 4 days. These treated cells were diluted to 10x10<sup>4</sup> cells/dish and cultured in D<sub>3</sub> free medium. NBT positive (●) and negative (○) cells were determined at indicated times.

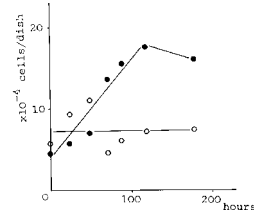
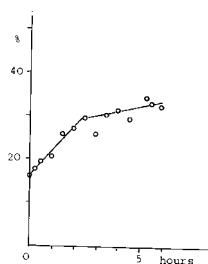


Fig. 3. b) The procedure was essentially the same as in Fig. 3a. Treated cells were transferred to a new medium containing D<sub>3</sub>. NBT positive (●) and negative cells (○) were determined.

In order to examine when NBT positive cells appeared in cell cycle, the following experiment was carried out. HL-60 cells were plated at a concentration of 2x10<sup>4</sup> cells/ml in a medium containing 25 nM D<sub>3</sub>. Two days later nocodazole (Sigma) was added at 0.05  $\mu$ g/ml and incubated for 14 hours to accumulate mitotic cells. After replacement with D<sub>3</sub> containing medium cell number and percentage of NBT positive cells were determined every 30 min. Both cell number and the percentage of NBT positive cells increased abruptly, indicating that NBT positive cells appeared after mitosis (Fig. 4).

Fig. 4. Effect of removal of nocodazole on the ratio of NBT positive to total cells. Cells were cultured for 2 days in the presence of 25 nM D<sub>3</sub>. Then nocodazole was added at a concentration of 0.05  $\mu$ g/ml and incubated for 14 hours. At 0 time the medium was replaced with only D<sub>3</sub> containing medium. At indicated times the ratios of NBT positive to total cells were determined.



Furthermore, cells (5000 cells/55mm dish) were inoculated into 0.35% soft agar containing 25 nM D<sub>3</sub>. Solitary cells were marked after solidification at several different locations. The

soft agar cultures were incubated for 4 days at 37 C and then overlaid with NBT solution to detect differentiated cells. As shown in Fig. 5, two cells were NBT negative and the other positive. This photograph was compatible with the above kinetic studies.

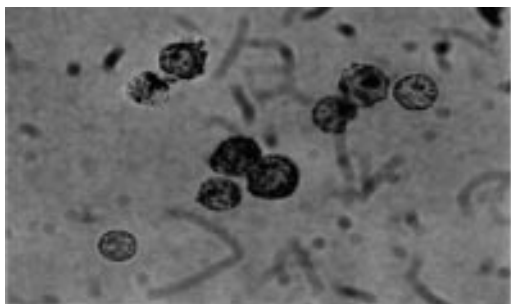


Fig. 5  
Production of NBT positive

ve cells in a soft agar. As a base layer one ml of 0.5% agar was plated in 55 mm petri dishes. After solidification 0.3 ml of 0.35% agar with 5000 cells was overlaid. Both the layers contained RPMI 1640 medium, 10% fetal bovine serum and 25 nM  $D_3$ . These dishes were incubated for days at 37 C and 1.3 ml of NBT solution was gently overlaid. Cells were stained at 37 C for 20 min. Colonies were observed with a Nikon Diaphoto microscope. x200

## DISCUSSION

In this study we examined how differentiated cells were produced in human promyelocytic leukemic cells, HL-60, which have been studied extensively ever since its establishment. We utilized  $D_3$  as a potent inducer and cellular superoxide production as the functional differentiation marker.

The highest ratio of NBT positive to total cells was 55% in this cell line at  $5 \times 10^{-8}$  M  $D_3$ . It was of interest that at concentrations higher than  $5 \times 10^{-8}$  M the ratio of NBT positive cells did not increase but leveled off. On the other hand, cell number started decreasing at more than  $5 \times 10^{-8}$  M  $D_3$ , namely these higher concentrations were toxic to HL-60 cells. In consideration of these results  $25 \times 10^{-9}$  M of  $D_3$  was the optimum concentration for experiments.

In the presence of 25 nM  $D_3$  cell growth became very slow on a logarithmic scale as compared with that of control culture. When HL-60 cells were inoculated at  $2 \times 10^4$  cells/ml and incubated for more than 100 hours, cells grew steadily and never flatted off on a logarithmic scale (data not shown). When growth curve of  $D_3$  treated cells was drawn on a normal scale, it became linear as shown in Fig. 1, which suggested that in the presence of  $D_3$  a certain portion of HL-60 cells might become non-dividing cells.

Considering that HL-60 cells grew linearly on a normal scale and the ratio of NBT positive to negative cells was linear in the presence of  $D_3$  (data not shown), absolute numbers of NBT positive and negative cells were calculated (Fig. 2). NBT negative cells increased about twice and then remained constant but NBT positive cells increase linearly during the experiment. It could be concluded that the linearly growing cells in Fig. 1 were NBT positive.

When NBT stained cells were observed microscopically, paired cells (NBT positive and negative) were quite often observed. These paired cells were not fortuitous but might be antecedents for single NBT positive cells. The ratio of paired to single positive cells were high during the early period of incubation in the presence of  $D_3$  (data not shown). These paired cells might be in the final phase of cytokinesis. We did not find these paired cells which were still in anaphase or telophase. Therefore, NBT positive cells appeared during the last period of cytokinesis. We were not able to detect all-NBT positive cells, indicating that NBT positive cells did not multiply anymore.

When HL-60 cells were cultured in the presence of  $D_3$ , NBT positive cells started to increase slowly in the first 40 hours and after then linearly. When cells which were treated for 4 days in  $D_3$  were placed in a new medium containing  $D_3$ , NBT positive cells began to grow linearly without showing any lag. This experiment showed that it took about one generation time to produce NBT positive cells

regularly. On the other hand, when  $D_3$  treated cells were placed in normal medium, NBT negative cells started to increase logarithmically.

In this study we demonstrated that NBT positive cells (namely, functionally differentiated cells) appeared after cell division, which did not necessarily imply that the presence of  $D_3$  was required just during the early period of G1 in cell cycle. It has been a matter of controversy whether or not DNA synthesis was necessary for differentiation (3, 16-18). It would be important to perform further experiment, considering that NBT positive cells are produced after mitosis.

Taking kinetic studies and soft agar experiment, we could propose the model of differentiation shown in Fig. 6. In control culture HL-60 cells multiply logarithmically (Fig. 6a). In the presence of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  cells multiply as shown in Fig. 6b. NBT negative cells function as so-called stem cells and produce positive cells which do not multiply. Using this model the growth curve of  $D_3$  treated cells would be linear on a normal scale.

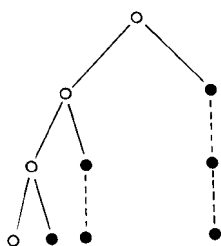


Fig. 6. a) Schematic presentation of logarithmic cell growth.

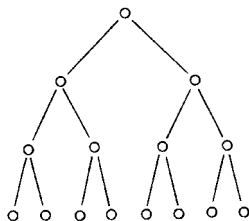


Fig. 6. b) Schematic presentation of hypothetical cell growth in the presence of  $D_3$ . Dividing cells (o) and differentiated cells (●).

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