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DOWN-REGULATION OF C-MYC PROTEIN EXPRESSION IN G0/G1 PHASE OF THE CELL CYCLE DURING THE INDUCTION OF DIFFERENTIATION BY 1, 25 DIHYDROXYVITAMIN D3 IN HL-60 CELLS

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It is well established that c-myc expression is down-regulated during induction of differentiation in various leukemia cell lines including HL-60 human myeloid leukemia cells. Previously we have shown that c-myc protein expression was specifically down-regulated in G0/G1 phase of the cell cycle during induction of granulocytic differentiation with dimetylsulfoxide (DMSO) in HL-60 cells. In the present study, we examined whether c-myc protein expression is also down-regulated in G0/G1 phase during monocytic differentiation of HL-60 cells. HL-60 cells can be induced to differentiate along monocytic lineage by treatment with 1, 25 dihydroxyvitamin D3 (D3). C-myc protein expression was down-regulated after 72h of treatment with D3 in HL-60 cells. Simultaneous analysis of protein level and cell cycle using a two-color flow cytometry showed that the down-regulation of c-myc protein expression occurred specifically in G0/G1 phase. Down-regulation of c-myc protein expression in G0/G1 phase was more clearly shown by using synchronized cell population that was arrested at G0/G1 phase by serum starvation. Furthermore, G0/G1-synchronized cells showed a higher susceptibility to differentiation in response to D3. These results suggest that c-myc protein is down-regulated in G0/G1-specific phase during not only granulocytic differentiation but also monocytic differentiation, and down-regulation of c-myc protein expression in G0/G1 phase is involved in the program of differentiation of HL-60 cells.

Key words: c-myc/differentiation/cell cycle/HL-60 cells

HL-60 human acute myeloid leukemia cells can be induced to terminally differentiate along either the granulocyte lineage with retinoic acid (RA) or dimetylsulfoxide (DMSO), or the monocyte-macrophage lineage with 1, 25 dihydroxyvitamin D3 (D3) or 12-O-tetradecanoylphorbol-13-acetate (TPA) (1). These terminally differentiated HL-60 cells subsequently die by apoptosis (2). Although the mechanisms inducing differentiation are not clear, several oncogene products are known to be involved in this process (1,3,4). Among them, c-myc protein is implicated in the processes of cell transformation, cell cycle progression, differentiation and apoptosis (1,3-16). C-myc protein is localized to the nucleus (5) and binds to the specific DNA sequence CACGTG (6). C-myc protein functions as a transcriptional factor and modulates the expression of target gene. During induction of differentiation of several human myeloid leukemia cell lines, c-myc transcript and protein level are down-regulated, and this down-regulation of c-myc expression has been reported to be necessary for the induction of differentiation (1,3,4,11-14). However, the relationship between down-regulation of c-myc expression and cell cycle remains unclear. Previously we have investigated the relationship between down-regulation of c-myc expression and cell cycle during induction of granulocytic differentiation with dimetylsulfoxide (DMSO) in HL-60 by using a two-color flow cytometry and showed that down-regulation of c-myc protein expression occurred in G0/G1-specific phase of the cell cycle (3). In the present study we further examined whether c-myc protein expression is also down-regulated in G0/G1 phase during monocytic differentiation of HL-60 cells by treatment with D3. Synchronized cell population was also used for assaying the effects of the protein expression in the cell cycle phases on the induction of differentiation. Here, we show that c-myc protein is also down-regulated in G0/G1-specific phase during monocytic differentiation induced by D3 in HL-60 cells.

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MATERIALS AND METHODS

Cell line and culture conditions

HL-60 cells were cultured in RPMI 1640 medium (Flow Laboratories) containing 10% heat-inactivated fetal calf serum (FCS) supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37℃ in a humidified atomsphere containing 5% CO_2. Unless otherwise indicated, the experiments were performed on exponentially proliferating HL-60 cells. The number of the cells in suspension culture was determined by standard leukocyte counting using trypan blue dye-exclusion method.

Assay of differentiation

Exponentially proliferating HL-60 cells at an initial cell density of 2×10^5cells/ml were treated with D3 (Chugai Pharmaceutical Co. Ltd.) for defined periods (0-72h). The optimal differentiation-inducing concentration of this agent was determined to be 10^{-7}M. Treated cells were cytocentrifuged, and May-Grü wald-Giemsa stain was performed to evaluate morphological differentiation. Nonspecific esterase (α-naphthyl acetate) stain was also performed to study the monocyte lineage-specific differentiation.

Antibodies

The anti-c-myc monoclonal antibody was obtained from Cambridge Research Biochemicals (Clone 6 E 10). This antibody is a mouse IgG1 obtained with a synthetic peptide comprising amino acids 171 to 188 of the c-myc protein (17). This antibody was used as the first antibody for c-myc staining, the MOPC-1 mouse IgG1 (Cappel) being used as a control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Becton Dickinson) was used as the second antibody in an indirect immunofluorescence method of flow cytometry applied.

Flow cytometric analysis

The level of c-myc protein expressed in HL-60 cells was analyzed by flow cytometry using an indirect immunofluorescence method, and the correlation between protein expressed and DNA content was analyzed by a two-color flow cytometric method in which the cells were simultaneously stained with anti-c-myc monoclonal antibody, and propidium iodide (PI, Sigma Chemical Co.) as previously described (3,4). After treatment of HL-60 cells with D3 for defined periods (0-72h), 2×10^6cells were harvested, washed twice with cold PBS, and fixed with 100% ethanol for 1h at 4℃. Then, the cells were permeabilized with PBS containing 0.05% Tween 20 and 0.5% bovine serum albumin (BSA) (PBS/0.05% Tween 20/0.5% BSA) for 5 min. The fixed, permeabilized cells were washed with cold PBS/0.05% Tween 20/0.1% BSA and incubated with anti-c-myc monoclonal antibody (1:160 dilution) or a control antibody (MOPC-1, equivalent dilution) for 1h at 4℃. After washing with cold PBS/0.05% Tween 20/0.1% BSA, the cells were incubated with FITC-conjugated anti-mouse IgG antibody (1:10 dilution) for 30 min at 4℃. Finally the cells were treated with PI (50 μg/ml) for 5 min at room temperature, and analyzed on a Coulter; EPICS flow cytometer. Excitation wavelenghth was 488nm, and the power output was 200mW. FITC-fluorescence signals were measured at 530nm through band pass filters and amplified logarithmically. PI-fluorescence signals were measured at 610nm and amplified logarithmically.

Synchronization of HL-60 cells at G0/G1 phase and induction of differentiation

Logarithmically proliferating HL-60 cells go to arrest at G0/G1 phase of the cell cycle under the condition of serum starvation. By preliminary experiments of effects of the synchronization at G0/G1 phase and the maintenance of cell viability, the optimal concentration of FCS and cultivation time were determined to be as 0.5% and 72h, respectively. The arrested or non-arrested cells at an initial cell density of 2×10^5cells/ml were treated with 10^{-7}M D3 in the medium containing 10% FCS. At defined periods the cells were harvested and effects of induction of differentiation were evaluated by morphological change and the expression levels of CD11b surface antigen (by flow cytometric method using anti-CD11b monoclonal antibody: Becton Dickinson). C-myc protein expression and DNA content were investigated by flow cytometry (described above).

RESULTS

Induction of monocytic differentiation by D3 in HL-60 cells

Treatment of HL-60 cells with D3 resulted in morphological changes showing characteristic of mature monocyte with an indented nucleus and abundant cytoplasm as shown in Fig 1. HL-60 cells that were treated with D3 for 72h showed cytoplasmic staining with non-specific esterase (data not shown), indicating that HL-60 cells can be induced to differentiate to monocytic lineage by D3 as previously reported (1).

Levels of c-myc protein during induction of monocytic differentiation in HL-60 cells

As shown in Fig. 2, in untreated HL-60 cells c-myc protein was constitutively expressed (almost 100% positive). Treated with D3 for 3h or 24h, the level of c-myc protein expression was not changed, however, at 72h of treatment, c-myc expression was down-regulated (Fig. 2).

Two-color flow cytometric analysis of c-myc protein and DNA content during induction of monocytic differentiation in HL-60 cells

To understand the correlation between levels of c-myc protein expression and the cell cycle, we examined the protein expression during cell cycle phases in HL-60 cells using a two-color flow cytometric method as pre-

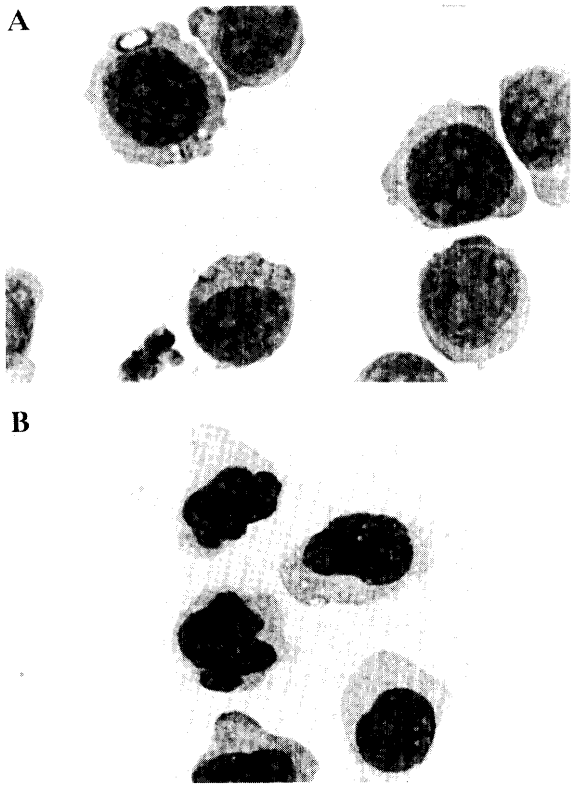

Fig. 1. Induction of monocytic differentiation by D3 in HL-60 cells. HL-60 cells were untreated (A) or treated with 10^{-7}M D3 for 72h (B). Photograph shows morphology of May-Giemsa stained cytocentrifuged HL-60 cells. At 72h of treatment a large number of the cells showed a morphologic change showing mature monocytes. (May-Giemsa stain; original magnification, × 1000)

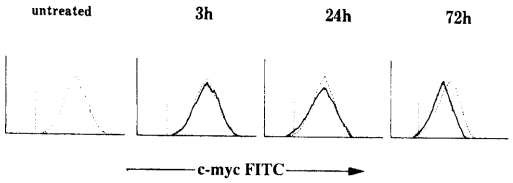

Fig. 2. C-myc protein expression during induction of differentiation in HL-60 cells. HL-60 cells were treated with 10^{-7}M D3 for the indicated times and the expression level of c-myc protein in untreated (*dashed* line; ·····) or treated cells (*bold* line; ——) was analyzed by flow cytometry. The vertical dashed line indicates the negative control level staining with MOPC-1 IgG1. This is one of three experiments with similar results.

viously described (3,4). As shown in Fig. 3A, in untreated cells c-myc protein was constitutively expressed thoughout the cell cycle phases, but a small population of G0/G1 cells expressed decreased level of the protein as compared with the cells in other cell cycle phases. At 72h of treatment, a large number of the cells were arrested in G0/G1 phase (Fig. 3B), and most of the cells treated with D3 were induced to differentiation at this time as shown in Fig. 1. This result is consistent with previous reports that terminally differentiated cells are arrested in G0/G1 phase (18,19). Importantly, c-myc protein expression was decreased in some population of these G0/G1-arrested cells (Fig. 3B), suggesting that c-myc protein expression was down-regulated in G0/G1 phase during monocytic differentiation of HL-60 cells induced by D3.

Regulation of c-myc protein expression in G0/G1 phase and induction of differentiation during treatment of G0/G1-arrested HL-60 cells with D3

Next, we investigated whether c-myc protein expression is also down-regulated in G0/G1-specific phase by using G0/G1-synchronized HL-60 cells. A large number of HL-60 cells underwent cell cycle-arrest at G0/G1 phase by cultivation with low concentration of FCS (0.5%). G0/G1-arrested cells showed greater down-regulation of c-myc protein expression as compared with the cells in other cell cycles (Fig. 4A). In addition, a small population of apoptotic cells which could be recognized as hypodiploid cells by DNA content were also observed. After treatment of these arrested cells with D3 for 24h, c-myc protein expression was further decreased in G0/G1 phase (Fig 4B). Moreover, these arrested cells became more adherent to the bottom of the plastic culture flask than non-arrested cells when treated with D3 for 72h (Fig. 5). Phenotypically the expression of CD11b antigen was also increased in arrested cells as compared with non-arrested cells (Fig. 6). These results indicate a higher susceptibility of G0/G1-arrested cells to differentiation. Furthermore, this ability of G0/G1-arrested cells to potentiate differentiation was consistent with the drastic down-regulation of c-myc protein expression in G0/G1 phase, suggesting that down-regulation of c-myc protein expression in G0/G1 phase is closely associated with induction of monocytic differentiation in HL-60 cells.

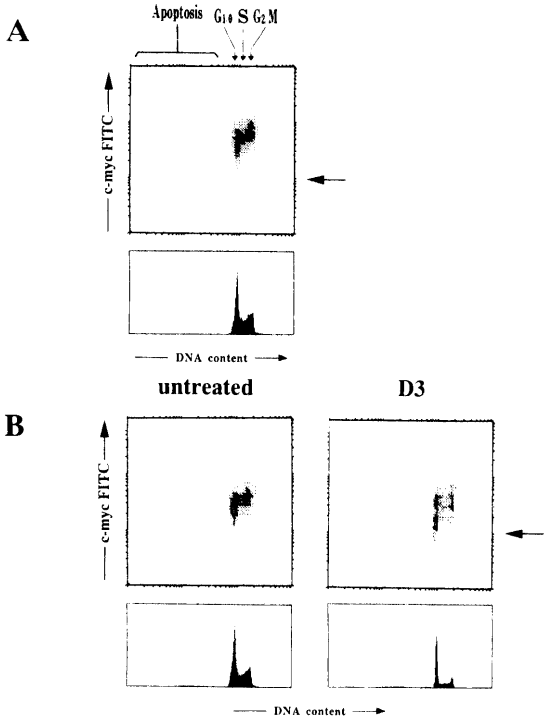

Fig. 3. Two-color flow cytometric analysis of c-myc protein expression and DNA contents during induction of differentiation in HL-60 cells. The correlation of c-myc protein expression and DNA contents in untreated cells (A) or treated cells with D3 for 72h (B) was analyzed by a two-color flow cytometry. The *arrow* at right side of the figures indicates the negative control levels for c-myc protein fluorescence intensity. This is one of three experiments with similar results.

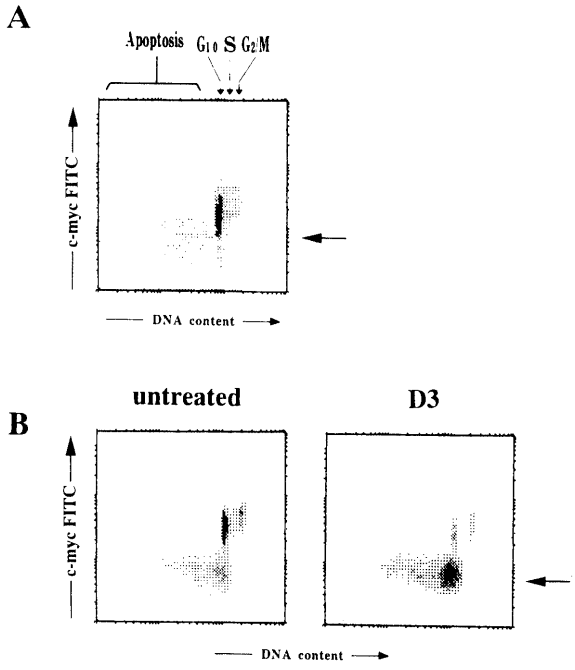

Fig. 4. C-myc protein expression in G0/G1-arrested HL-60 cells during induction of differentiation. The cells were arrested at G0/G1 phase by cultivation with 0.5% FCS for 72h. The correlation of c-myc protein expression and DNA contents in arrested cells (A) or arrested cells that were treated with D3 for 24h (B) was analyzed by a two-color flow cytometry. The *arrow* at right side of the figures indicates the negative control levels for c-myc protein fluorescence intensity. Similar results could be obtained by a second independent experiment.

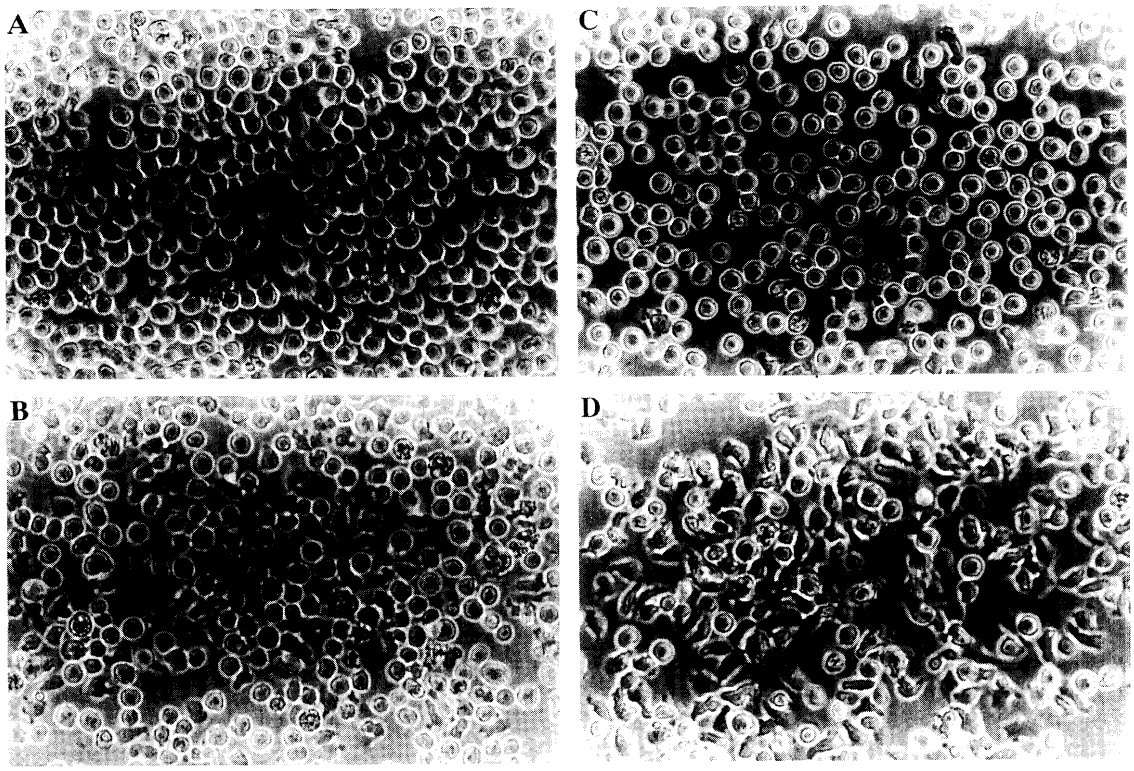

Fig. 5. Morphological changes induced by D3. Non-arrested (A, B) or arrested (C, D) HL-60 cells were cultured for 72h in the absence (A, C) or presence (B, D) of D3 and were photographed by phase-contrast microscopy. A second independent experiment yielded similar results.

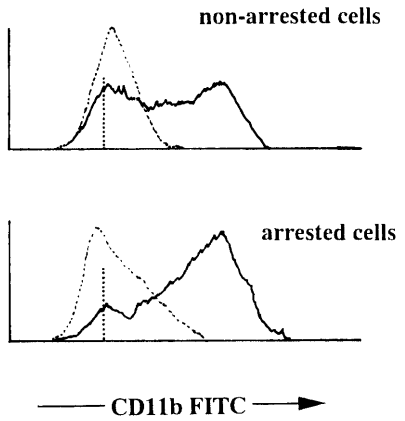

Fig. 6. CD11b expression induced by D3. Non-arrested (upper panel) or arrested (lower panel) HL-60 cells were cultured for 72h in the absence (*dashed* line; ····) or presence (*bold* line; ——) of D3, and the induction of CD11b surface antigen expressed on the cells was analyzed by flow cytometry. The vertical dashed line indicates the negative control level.

DISCUSSION

C-myc protein functions as a growth promoting factor that especially exerts its role during G1 transit of various types of cells (10). Expression of c-myc protein is also associated with the progression of transformation in many tumors (7-9). In contrast, differentiation together with apoptosis are growth-suppressive processes in which c-myc protein expression is inhibited (1, 3, 4, 11-14). We have previously shown that c-myc protein expression was rapidly down-regulated during granulocytic differentiation of HL-60 cells when treated with DMSO (4). In this study, down-regulation of c-myc protein expression occurred as early as 3h of treatment. Our result presented here also showed that c-myc protein expression was down-regulated during monocytic differentiation of HL-60 cells when they were treated with D3, although the down-regulation occurred at 72h of treatment. This is in accordance with a previous report indicating that the decrease of c-myc transcriptional levels in HL-60 cells during induction of monocytic differentiation with D3-treatment was slower than that during granulocytic differentiation with DMSO, and the expression of c-myc mRNA became undetectable after treatment with DMSO and D3 for 1h and 72h, respectively (13). This difference may be due to the differences between the mechanisms of granulocyte- and monocyte-lineage commitment.

Two-color flow cytometric analysis revealed that a large number of HL-60 cells treated with D3 were arrested in G0/G1 phase at 72h and c-myc protein expression was decreased in some population of these G0/G1-arrested cells. By using G0/G1-synchronized cell population of HL-60 cells it was more clearly shown that c-myc protein expression was down-regulated in G0/G1-specific phase during induction of monocytic differentiation with D3. These results suggest that c-myc protein is down-regulated in G0/G1-specific phase during not only granulocytic differentiation but also monocytic differentiation in HL-60 cells.

The role of down-regulation of c-myc protein in G0/G1 phase for induction of differentiation remains unclear. We showed here that G0/G1-synchronized cells took advantage for differentiation to non-synchronized cells and this advantage was associated with drastic

down-regulation of c-myc protein expression. In addition, the previous reports have shown that expression of c-myc in G0/G1 phase allows cells to progress the cell cycle without the initiation of differentiation pathway (10,20). From these results it is suggested that down-regulation of c-myc protein expression in G0/G1 phase is important for cellular differentiation. A physiological role of G0/G1 phase for regulating induction of differentiation is also recognized as the checkpoint to determine the fate of cells to enter cell cycle or differentiate (20). Regarding this property, both cell cycle arrest and down-regulation of c-myc protein may be important events mediating differentiation programs in HL-60 cells. This is a very beneficial suggestion for the clinical application of induction of differentiation. The combination treatment with differentiation-inducer and some kind of anticancer agents providing the down-regulation of c-myc expression and/or G0/G1-arrest may be useful in terms of the enhancement of clinical efficacy of differentiation-induction therapy. Differentiation-induction therapy is now one of the treatments for human leukemias and/or cancers.

In conclusion, our results suggest that c-myc protein is down-regulated in G0/G1-specific phase during not only granulocytic differentiation but also monocytic differentiation in HL-60 cells. G0/G1-specific down-regulation of c-myc protein expression may be an important event for the determination of the fate of cells to undergo differentiation. Further studies should be required to understand the cellular and molecular mechanisms regulating differentiation.

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