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# REGULATION OF BCL-2 PROTEIN EXPRESSION BY C-MYC PROTEIN IN HL-60 CELLS DURING THE INDUCTION OF DIFFERENTIATION-ASSOCIATED APOPTOSIS

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Oncogene products, c-myc and bcl-2 protein, play an important role in the decision of the fate of cells, such as proliferation, differentiation and apoptosis. Terminally differentiated HL-60 human leukemia cells express low levels of both c-myc and bcl-2 protein, and die by apoptosis. We have previously reported that down-regulation of c-myc protein was sufficient for the induction of apoptosis in HL-60 cells, while down-regulation of bcl-2 protein was not the case. To understand regulatory mechanism between c-myc and bcl-2 protein expression, we examined the effects of c-myc or bcl-2 antisense oligonucleotides on the expression levels of bcl-2 or c-myc protein, respectively. Our experiments showed that c-myc antisense down-regulated bcl-2 protein expression, whereas bcl-2 antisense did not change the levels of c-myc protein expression. These results suggest that bcl-2 protein is regulated sequentially at downstream of the decrease in c-myc protein expression during the process of differentiation induction program. Our study identified c-myc protein as a possible primary candidate rather than bcl-2 in the gene targeting therapy of leukemia.

Key words: c-myc, bcl-2, differentiation, apoptosis, HL-60 cells

## INTRODUCTION

Terminally differentiated hematopoietic cells have a short life-span and subsequently undergo apoptotic cell death (1, 2). We have previously reported that terminally differentiated HL-60 human leukemia cells die by apoptosis, closely associated with marked down-regulation of both c-myc and bcl-2 protein expression (3, 4). Although c-myc gene is usually implicated in cell-cycle progression, its central role in the induction of apoptosis is also demonstrated (5). These opposing roles of c-myc protein in the determination of cell fate require the presence of modifying gene whose products block apoptosis at the tumorigenesis. Thus, cooperative interaction between c-myc and bcl-2 protooncogenes, in which bcl-2 specifically abrogates c-myc-induced apoptosis without affecting the c-myc mitogenic function, has been proposed (6). This proposal raises the question whether down-regulation of bcl-2 alone could induce the apoptosis of HL-60 cells, which express constitutive bcl-2 and amplified c-myc proteins. We have previously demonstrated that bcl-2 antisense oligonucleotides did not induce apoptosis in HL-60 cells during the early time of treatment, whereas c-myc antisense could induce apoptosis (3). This result further asks the relationship between the c-myc and bcl-2 protein in the apoptosis induction program. To address this, we examined the effects of c-myc or bcl-2 antisense oligonucleotides on the expression levels of bcl-2 or c-myc protein, respectively. Both protein expressions were investigated by flow cytometry using an indirect immunofluorescence method, in which HL-60 cells were specifically stained by monoclonal antibodies against c-myc and bcl-2 protein.

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## MATERIALS AND METHODS

### *Cell culture*

HL-60 cells were cultured in RPMI 1640 medium (Flow Laboratories) containing 10% heat-inactivated fetal calf serum supplemented with penicillin (100

IU/ml) and streptomycin (100 mg/ml) at 37°C in a humidified atomsphere containing 5% $CO_2$. The experiments were performed on exponentially proliferating HL-60 cells.

***Antibodies***

Anti c-myc protein antibody (IgG1), a mouse monoclonal antibody to human c-myc protein, was purchased from Cambridge Research Biochemicals (Clone 6E10). Anti-bcl-2 monoclonal antibody was also purchased from Cambridge Research Biochemicals (Clone 124; a mouse IgG1). Mouse IgG1, which was used as a control to the anti c-myc and bcl-2 protein antibodies, was obtained from Cappel (MOPC 21). These reagents were dissolved in physiologic saline and stored until use at -20°C. Fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG was purchased from Becton Dickinson.

***Flow cytometric analysis***

The levels of c-myc and bcl-2 protein expression were analyzed by flow cytometry using an indirect immunofluorescence method, as previously described (3, 4, 7). In this method, $2x10^6$ treated HL-60 cells were fixed with 100% ethanol for 1 h at 4°C and permeabilized with phosphate-buffered salines (PBS) containing 0.05% Tween 20 and 0.5% bovine serum albumin (BSA). Then, the cells were incubated with anti-c-myc monoclonal antibody (1:160 dilution), anti-bcl-2 monoclonal antibody (1:80 dilution) or a control antibody (MOPC-1, equivalent dilution, respectively) for 1 h at 4°C. The cells were then washed with cold PBS/0.05% Tween 20/0.1% BSA and incubated with FITC-conjugated anti-mouse IgG antibody (Becton Dickinson, 1:10 dilution) for 30 min at 4°C. Finally the cells were analyzed on a Coulter; EPICS flow cytometer. Excitation wavelength was 488 nm, and the power output was 200 mW. FITC-fluorescence signals were measured at 500 nm through band-pass filters and amplified logarithmically.

***c-myc and bcl-2 antisense oligonucleotides***

Sense and antisense 18-mer nuclease-resistant phosphorothioate oligodeoxynucleotides directed to translation initiation site of c-myc or bcl-2 transcripts were prepared using an Applied Biosystems (Foster City, CA, USA) 392 DNA/RNA synthesizer. The sequences of oligonucleotides were; 5'-CCCCTCAACGTTAGCTTC-3' (sense) and 5'-GAAGCTAACGTTGAGGGG-3' (antisense) (codons 2-7) for c-myc, and 5'-GCGCACGCTGGGAGAACG-3' (sense) and 5'-CGTTCTCCCAGCGTGCGC-3' (antisense) (codons 2-7) for bcl-2. These oligonucleotides were purified by Sephadex G-25 (DNA grade) gel filtration chromatography, and then the purity was checked by re versed-phase high-performance liquid chromatography. HL-60 cells were cultured with 50 μg/ml of c-myc or bcl-2 sense or antisense oligonucleotides for 48 h and expression levels of c-myc and bcl-2 protein were analyzed by flow cytometric method as described above.

## RESULTS AND DISCUSSION

As shown in Figure 1, exposure of HL-60 cells to c-myc antisense resulted in down-regulation of bcl-2 protein expression, however, c-myc sense did not alter bcl-2 protein expression. On the other hand, both bcl-2 sense and antisense did not change the levels of c-myc protein expression. Since we demon-

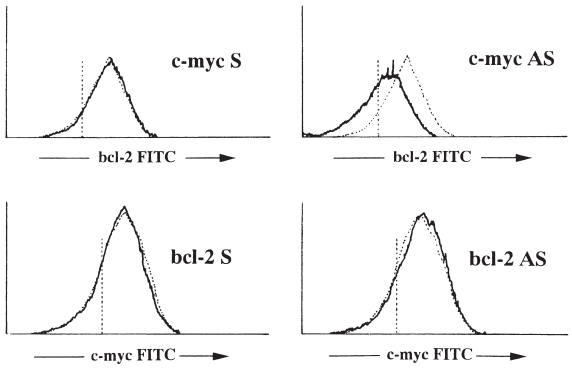


Fig. 1. Effects of sense or antisense oligonucleotides on c-myc or bcl-2 protein expression. HL-60 cells were exposured to c-myc or bcl-2 sense (S), or antisense (AS) oligonucleotides for 48 h. After the harvest, c-myc or bcl-2 protein expression was analyzed by flow cytometry using an indirect immunofluorescence method. Both oncoproteins were constitutively stained with specific monoclonal antibodies in the control culture cells (*the dotted curve:* ·····) as compared with negative control staining with MOPC-1 $IgG_1$ (the vertical dotted line). The *solid curve* ( - ) corresponds to the fluorescence intensity of the cells stained with monoclonal antibodies against c-myc or bcl-2 protein after the treatment with S or AS oligonucleotides.

strated the antisense-specific decrease in expression of each oncoprotein previously (3), our results presented here suggest that inhibition of c-myc protein expression mediates down-regulation of bcl-2 protein expression in HL-60 cells. Although c-myc is known as transcription factor, there is no evidence that bcl-2 is the target gene of its transcriptional activity.

It is well established that HL-60 cells are induced to terminally differentiate along granulocytic pathway with retinoic acid or dimetylsulfoxide, or monocytic/macrophagic pathway with 1,25-dihydroxyvitamine D3 or tumor necrosis factor-α (8). During treatment of HL-60 cells with either of these inducers, c-myc protein was rapidly down-regulated as early as 3 h of treatment, which was followed by down-regulation of bcl-2 protein after 24 h (3, 4). When the cells were terminally differentiated (at 72 h), both proteins were markedly down-regulated and apoptosis was subsequently induced (3). Thus, down-regulation of both c-myc and bcl-2 protein expression is specifically involved in the induction of differentiation-associated apoptosis in HL-60 cells. Taken together, down-regulation of c-myc protein expression may be differentiation-specific molecular event at the initial phase of differentiation program. Our results presented here suggest that down-regulation of bcl-2 protein observed during terminal differentiation of HL-60 cells is, at least in part, triggered by the down-regulation of c-myc protein, namely, the sequential regulation between c-myc and bcl-2 protein in the induction of differentiation-associated apoptosis of HL-60 cells.

On the other hand, we have previously demonstrated that serum-depleted or over-growth cultivation resulted in the induction of apoptosis in HL-60 cells (3). These apoptotic cells expressed the decreased levels of c-myc protein while the expression of bcl-2 protein remained at relatively high levels, suggesting that bcl-2 expression is affected by various pathways and that down-regulation of bcl-2 protein via c-myc can be prevented by other anti-apoptotic signal. Further examinations should be needed to clarify the molecular mechanism regulating these oncogenes.

In this study, we suggest a regulatory mechanism of bcl-2 expression by c-myc protein during the process of differentiation. Recognition of this regulatory mechanism of these oncogene products is very important in terms of the gene targeting as the new field of leukemia therapy, in which suppression of bcl-2 alone is not enough for the induction of apoptosis, but suppression of c-myc might be rational for the targeting therapy.

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