Alterations of c-myc Expression by Antisense Oligodeoxynucleotides Enhance the Induction of Apoptosis in HL-60 Cells

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ABSTRACT

This study was designed using c-myc antisense oligodeoxynucleoside phosphorothioate (AS PS-ODN) to evaluate how alterations of c-myc expression in HL-60 human myeloid leukemia cells could influence the induction of apoptosis. Unexpectedly both the continuous down-regulation of c-myc expression caused by exposure to c-myc AS PS-ODN and up-regulation after its withdrawal influenced apoptosis. We found that continuous suppression of c-myc expression by 10 μM c-myc AS PS-ODN could decrease the proliferation of HL-60 cells to approximately 60% of the control growth after 3 days of suspension culture, and when assessed morphologically the percentage of cells undergoing apoptosis was 3.5%. Evidence either of cell cycle arrest or cell cycle prolongation was not detected. It is likely that apoptosis induced by the sustained down-regulation of c-myc expression with AS PS-ODN treatment was solely sufficient to explain the inhibition of cell proliferation. Up-regulation of c-myc expression was observed within 1 h after c-myc AS PS-ODN withdrawal. This up-regulation further enhanced induction of apoptosis and involved up to 32% of the cells. These results suggest that while the continuous suppression of c-myc expression caused a constant effect on the induction of apoptosis, its abrupt up-regulation could rapidly drive a considerable number of HL-60 cells into the apoptotic pathway.

INTRODUCTION

The c-myc proto-oncogene is evolutionarily conserved in all vertebrates and is constitutively expressed throughout the cell cycle in most normal, dividing cells and in malignant cells as well (1, 2). The HL-60 cell line, derived from a patient with acute myeloid leukemia, contains multiple copies of the c-myc gene (3). The relationship between c-myc expression and apoptosis was described by Wyllie et al. (4) who observed an increased rate of apoptotic death in fibroblasts transformed by c-myc oncogene. The c-myc protein was also shown to induce apoptosis in Rat-1 fibroblasts (5). Apparently conflicting results have also been reported. AS PS-ODN corresponding to c-myc can block c-myc expression in T-cell hybridomas and interfere with all aspects of activation-induced apoptosis (6). Moreover, glucocorticoid causes apoptosis of the 697 pre-B lymphocytes by repressing the expression of c-myc (7), and interleukin 6-induced growth inhibition and apoptosis in Y6 cells are preceded by the down-regulation of c-myc gene expression (8). Therefore, it is likely that enforced alterations of c-myc expression either by AS PS-ODN (6) or by the retroviral c-myc insertion (4, 5) may result in the induction of apoptosis.

In the present study, we have investigated how cell proliferation and the induction of apoptosis were affected by the c-myc protein levels using c-myc AS PS-ODN. It has been reported that unmodified phosphorodiesters c-myc AS ODN reduced c-myc expression and inhibited the proliferation of HL-60 cells (9, 10). PS-ODNs, in which a nonbridging oxygen atom is substituted by a sulfur atom, retain the property of high water solubility and the ability to elicit RNase H activity and are generally nuclease-resistant. This allowed us to observe the effects on the proliferation and the induction of apoptosis by down- and subsequent up-regulation of c-myc expression using c-myc AS PS-ODN.

MATERIALS AND METHODS

Cell Line. The HL-60 myeloid leukemic cell line was kindly provided by Dr. Jun Minowada (Hayashibara Fujisaki Cell Center, Okayama, Japan) and maintained in suspension cultures in RPMI 1640 (Flow Laboratories, Irvine, United Kingdom) supplemented with 10% heat-inactivated FCS (Flow Laboratories, McLean, VA) at 37°C in a fully humidified atmosphere of 5% CO2 in air. Cells in exponential growth were used in this study.

Preparation of PS-ODNs. PS-ODNs were synthesized by cyanoethylphosphoramidite chemistry on an automated DNA synthesizer (Model 381A; Applied Biosystems, Foster City, CA). Sulfurizing reagent, tetraethylthiuram disulfide (Applied Biosystems), was used according to the instructions of the manufacturer. AS and S ODNs targeting the translation starting site of c-myc mRNA were synthesized. The sequence of 15-mer c-myc AS PS-ODN was 5'-AACGTTGGGGCCAT-3'. The corresponding S sequence was used as a control. The solution containing the crude PS-ODNs was evaporated in vacuum and dissolved in 25 mM TEAB, and then the solution was loaded on a reverse phase C18 polymer column (MR-300; Shinya Chemical Industries, Ltd., Kyoto, Japan). After a washing with 25 mM TEAB buffer (pH 7.0) and 15% CH3CN, the elution was treated with 3% TFA in 100 mM TEAB, the support was eluted with 1% trifluoroacetic acid (Wako Pure Chemical, Osaka, Japan) for 5 min at RT, followed by immediate washing with 100 mM TEAB. PS-ODNs were eluted with 20% CH3CN in 100 mM TEAB, and the eluant was evaporated to dryness. The residue was dissolved in 100 mM triethylammonium acetate supplemented with 5% CH3CN and analyzed for purity using reverse phase liquid chromatography (Pharmacia, Uppsala, Sweden).

Suspension and Clonal Cultures. At initiation of culture, PS-ODNs were added directly to the cell suspension (5 × 10⁴ cells/ml), and the cell number and viability were evaluated every 24 h by trypan blue dye exclusion. After incubation of HL-60 cells with or without PS-ODNs for 24 h, an aliquot of culture medium containing leukemic cells was prepared in 1-ml volumes of DMEM containing 1% FCS and 5% CH3CN and analyzed for purity using reverse phase liquid chromatography. After 24 h, some cultures were centrifuged, and the pellets were resuspended in 0.5 ml of buffered formalin acetone for 5 s at RT and rinsed twice with PBS. The fixed cells were incubated with a saturating concentration of the anti-c-myc murine MAb, MYC-1, kindly provided by Dr. Hiroshi Shiku (Department of Oncology, Nagasaki University, School of Medicine, Nagasaki, Japan) (13). Cells stained with purified normal mouse IgG1 (Chemicon International, Inc., Temecula, CA) at 37°C in a fully humidified atmosphere of 5% CO2 in air. Colonies (>50 cells) were scored using a stereo zoom dissection microscope as described elsewhere (11).

Detection of c-myc Protein. Translation of c-myc mRNA into p56 (12) was detected by an indirect immunofluorescence method. Untreated, c-myc AS-treated, and S PS-ODN-treated HL-60 cells were centrifuged, and the pellets were resuspended in 0.5 ml of buffered formalin acetone for 5 s at RT and rinsed twice with PBS. The fixed cells were incubated with a saturating concentration of the anti-c-myc murine MAb, MYC-1, kindly provided by Dr. Hiroshi Shiku (Department of Oncology, Nagasaki University, School of Medicine, Nagasaki, Japan) (13). Cells stained with purified normal mouse IgG1 (Chemicon International, Inc., Temecula, CA) were used as a negative control. After 60 min on ice, the cells were washed with PBS three times and incubated with FITC-conjugated goat anti-mouse antibody [Fab(α′)] fractions of IgG; Cappel, Durham, NC). After three washings with PBS, the cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PS-ODN, phosphorothioate oligodeoxynucleotide; AS, antisense; S, sense; TEAB, triethylammonium bicarbonate; RT, room temperature; TdT, terminal deoxynucleotidyl transferase; BrdUrd, bromodeoxyuridine.
Intracellular c-myc protein was detected in a cytoplasmic preparation for immunocytochemical staining using the Histostain-SAP kit (Zymed Laboratories, Inc., San Francisco, CA). Untreated, c-myc AS PS-ODN-treated, and S PS-ODN-treated HL-60 cells were fixed with 4% formaldehyde (Nacalai Tesque, Kyoto, Japan) for 10 min and rinsed with PBS. Normal human peripheral lymphocytes were also prepared as a negative control. These preparations were incubated with MYC-1 for 4 h. Normal mouse IgG1 was used as a negative control for the first antibody. The cells were rinsed with PBS, incubated with biotinylated goat anti-mouse IgG antibody (Zymed) for 10 min, and then rinsed again with PBS. Thereafter, they were incubated with an enzyme conjugate for 10 min, rinsed with PBS, and incubated with the substrate-chromogen mixture.

Measurement of Differentiation Markers. Cell morphology was evaluated on a cytocentrifuge preparation following by methanol fixation and Wright-Giemsa staining. Apoptotic cells were identified morphologically according to proposed criteria (14). A minimum of 500 cells were examined per slide. Cytotoxic reactions for naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase were performed by standard methods described elsewhere. Alterations of the surface phenotype of HL-60 cells were examined by an indirect immunofluorescence method using MAb CD11b, CD13, and CD14. At least 1 × 10^6 cells were then analyzed using flow cytometry.

Detection of Apoptotic Cells Using Light Scattering Properties. Cells undergoing apoptosis display changes in morphology that can be detected by comparing the forward and side scatter profiles (15). Briefly, the cells were centrifuged and then the pellets were fixed in 70% ethanol at −20°C for at least 30 min. The cells were rinsed twice with PBS and their altered light scattering properties were analyzed by flow cytometry.

Detection of DNA Strand Breaks. To detect DNA strand breaks of DNA, a TdT assay (16) was used with some modifications. Briefly, HL-60 cells were fixed in suspension in PBS containing 1% formaldehyde on ice for 15 min, rinsed once with PBS, and then fixed in 70% ethanol at −20°C for 24 h. The cells were then centrifuged, rinsed twice with PBS, and resuspended in 50 μl of a buffer solution containing 0.2 M potassium cacodylate (Boehringer Mannheim, Mannheim, Germany), 2.5 mM Tris-HCl (pH 6.6), 2.5 mM cobalt chloride, 5 units of TdT (Boehringer Mannheim), 0.25 mg/ml of BSA (Sigma Chemical Co., St. Louis, MO), and 4 μM digoxigenin dUTP (Boehringer Mannheim). The cells were incubated in this solution at 37°C for 30 min, rinsed with PBS, and then resuspended in 100 μl of a solution containing SSC buffer (0.6 M NaCl-0.06 M sodium citrate; Sigma), 0.2 μg/ml of FITC-conjugated anti-digoxigenin antibody (Boehringer Mannheim), 0.1% Triton X-100 (Sigma), and nonfat dry milk (Dainihon Co., Ltd., Tokyo, Japan). After 30 min of incubation at RT in the dark, the cells were rinsed with PBS and analyzed by flow cytometry.

DNA Extraction and Agarose Gel Electrophoresis. Untreated and AS PS-ODN-treated HL-60 cells were resuspended in a lysis buffer and then in 500 μl of 10 mM Tris-HCl-1 mM EDTA-0.1 mM NaCl plus 100 μl of 10% SDS (Wako) and 1 μl of RNase (10 mg/ml; Sigma) and then incubated at 55°C for 30 min. After addition of 5 μl of proteinase K (10 mg/ml; Wako), tubes were incubated at 37°C for overnight. Once centrifuged after addition of 200 μl of 5 M NaCl, the upper layer was collected and isopropyl alcohol was added. Precipitated DNA was put into chilled ethanol, centrifuged for 30 min, and then resuspended in 10 mM Tris-HCl-1 mM EDTA. DNA samples, 0.2 μg each, were electrophoretically separated on 2% agarose gel containing ethidium bromide.

Cell Cycle Analysis. The DNA content of untreated and PS-ODN treated cells was determined by flow cytometry (17). Cells (3 × 10^6) were incubated in culture medium with 10 μM BrdUrd (Sigma) at 37°C for 10 min. FITC-conjugated MAB against BrdUrd (Becton Dickinson) was bound to the BrdUrd incorporated in DNA; then the cells were stained with propidium iodide (5 μg/ml; Sigma). At least 2 × 10^6 cells were subjected to replicate analyses. The stathmokinetic study was performed by administration of demecolincine (0.02 μg/ml; Sigma) for 12 h with or without PS-ODNs.

RESULTS

Inhibition of Cell Proliferation and Colony Formation by HL-60 Cells Using c-myc AS PS-ODN. The growth of HL-60 cells could be suppressed dose dependently by c-myc AS PS-ODN with a range from 5 to 20 μM, while it could not be significantly suppressed by 20 μM c-myc S (Fig. 1A). Similar dose dependency could be seen in three separate experiments. Clonal growth of HL-60 cells could be evaluated by the colony formation in agar. An aliquot of medium containing HL-60 cells treated with either c-myc AS or S for 24 h in suspension culture, when the cell numbers were almost equal, was put into agar culture without PS-ODNs and then incubated for 7 days. Colony formation of c-myc AS-treated HL-60 cells was suppressed to approximately 15% of control colonies, while no significant decrease was noted in cells treated with c-myc S PS-ODN (Fig. 1B). Similar suppression was seen in a separate experiment.

Evaluation of c-myc Protein Level. Suppression of c-myc protein synthesis after 12 h exposure to 5 μM c-myc AS PS-ODN was observed (Fig. 2A). In contrast, the c-myc protein level of 20 μM c-myc S-treated cells was equal to that of untreated cells (data not shown). Next, after exposure to c-myc AS PS-ODN for 12 h, HL-60 cells were washed to remove AS PS-ODN from medium and then cultured in AS-free RPMI 1640 plus 10% FCS for 1 h. C-myc protein level returned to that of untreated cells within 1 h. Untreated and S-treated cells revealed no alteration of c-myc protein level before and after medium change (Fig. 2B). A small peak with less fluorescence intensity was evident 6 h after AS withdrawal (Fig. 2C). This peak may represent cells undergoing apoptosis, where c-myc protein levels were further decreased due to cell degradation. Untreated and S-treated cells were heavily stained with MYC-1 in their nuclei, while those of AS-treated cells were significantly less stained (Fig. 2, D and E).
Fig. 2. Alterations of c-myc protein levels in HL-60 cells treated with c-myc AS PS-ODN. In A, after 12 h treatment with c-myc AS PS-ODN, c-myc protein levels were significantly less than levels in untreated cells. Bold line and shaded area, fluorescence activity of c-myc AS PS-ODN-treated and untreated cells, respectively. **--** **--**, untreated cells stained with normal mouse IgG1 as a negative control. In B, 1 h after c-myc AS PS-ODN withdrawal and washing of cells, the fluorescence intensity of AS-treated cells returned to same level as untreated cells. In C, after 6 h of AS withdrawal, c-myc AS PS-ODN-treated cells revealed a small peak with less fluorescence (open arrow). Except for this small peak, the c-myc protein level of AS-treated and untreated cells were essentially the same. Nuclei of untreated cells were heavily stained with MYC-1 MoAb (D), while those of c-myc AS PS-ODN-treated cells were significantly less stained (E).

Detection of Apoptotic Cells. After 12 h of exposure to c-myc AS PS-ODN, apoptotic cells were evident morphologically as 3.5 ± 1.3% (SD), whereas untreated or S-treated cells contained 0.4 ± 0.1% and 0.5 ± 0.1%, respectively. These percentages did not appear to be altered with longer incubation times. Intriguingly, HL-60 cells treated with c-myc AS PS-ODN for 12 h and then cultured in AS-free medium for 6 h caused a pronounced increase in the amount of apoptotic cells (Fig. 3). We confirmed this increased level of apoptosis using flow cytometric and electrophoretic analysis. Irrespective of exposure time to c-myc AS PS-ODN (for either 12 h or 3 days), the number of cells with lower forward scatter or having DNA strand breaks by TdT assay was consistently approximately 3% (Fig. 4, A and C). However, approximately 30% of HL-60 cells treated with c-myc AS PS-ODN for 12 h followed by culture in AS-free medium for 6 h were found to be apoptotic by the flow cytometric assay and were positive with TdT assay (Fig. 4, B and D). The percentages of apoptotic cells assessed morphologically were also 32 ± 5% (Fig. 4E). Gel electrophoresis of DNA extracted from these cells revealed a DNA ladder, characteristic of apoptosis (Fig. 5) (18). Despite the emergence of these apoptotic cells, the population of HL-60 cells with CD11b, CD13, and CD14 surface phenotypes was not significantly altered by exposure to c-myc AS PS-ODN when
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Fig. 3. Wright-Giemsa-stained HL-60 cells exposed to c-myc AS PS-ODN for 12 h followed by incubation in AS-free medium for subsequent 6 h.

Assessment of Cell Cycle. Cell cycle analysis revealed that after exposure to 10 μM c-myc AS PS-ODN for 12 h, 3.3 ± 1.6% of the cells appeared in the hypodiploid and near-triploid regions without incorporation of BrdUrd, while untreated or S-treated cells scarcely had such fraction (untreated, 0.8 ± 0.17%; S treated, 1.0 ± 0.13%) (Fig. 6). These regions are considered to represent apoptotic cells (19). Cell cycle analysis of HL-60 cells treated with c-myc AS PS-ODN was not significantly different from those found in untreated and S-treated cells. Furthermore, no cell cycle arrest at the G/SD boundary was observed (Table 1). The cumulative rates for 12 h of cell entrance into mitosis of untreated, AS-treated, and S-treated cells were 39.2, 42.1, and 43.3%, respectively.

DISCUSSION

The relationship between apoptosis and alterations in c-myc expression has been extensively studied in the past. However, no general agreement has yet been achieved regarding the significance of c-myc expression on the cell proliferation and the induction of apoptosis. Some investigators found that up-regulation of c-myc expression caused apoptosis (4-6, 20, 21), while others suggested that down-regulation may be mandatory for the induction of apoptosis (7, 8). The present study examined the effects on the proliferation and the induction of apoptosis by down- and up-regulation of c-myc expression in cultures of HL-60 cells by continuous exposure to and withdrawal of c-myc AS PS-ODN, respectively.

Consistent with previous reports, we confirmed the inhibitory effects of c-myc AS PS-ODN on the proliferation of HL-60 cells. However, we could not observe a significant induction of differentiation, although Holt et al. (10) reported that a granulocytic differentiation of HL-60 cells, albeit weak, could be induced by phosphodiester c-myc AS ODN, and also mentioned that the proportional prolongation of all phases of cell cycle resulted in the growth suppression. We have shown that there were no significant changes in cell cycle. Furthermore, the stathmokinetic study has revealed that the rate of cell entrance into mitosis of AS-treated HL-60 cells did not differ from that of untreated and S-treated cells. These reports together with our morphological observation of the emergence of a small percentage of apoptotic HL-60 cells by c-myc AS PS-ODN led us to investigate the mechanisms of inhibitory effects on their growth.

Since no significant induction of differentiation and no obvious cell cycle arrest or cell cycle prolongation were found in HL-60 cells treated with c-myc AS PS-ODN, we assumed that cells induced to the apoptotic pathway resulted directly in the growth suppression observed with these cell populations. Clonal growth of AS-treated HL-60 cells in agar was significantly suppressed before there was evidence of reduced cell growth in suspension culture. This may suggest that HL-60 cells undergoing apoptosis lost their self-renewal ability and could not contribute to the cell proliferation.

Evan et al. (5) reported using Rat-1 fibroblasts with constitutive c-myc expression that the interval between apparent morphological normality of cells undergoing apoptosis and complete cell fragmentation is typically about 30 min and, consistent with the observations...
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Fig. 5. DNA analysis by 2% agarose gel electrophoresis of the genomic DNA extracted from HL-60 cells treated with c-myc AS PS-ODN for 12 h followed by incubation in AS-free medium for a subsequent 6 h.

reported here, cell death continues at a more or less constant rate. If apoptotic cells appear at a constant rate, the growth rate of cells can be calculated as

\[
\text{Growth rate} = \left(1 - \frac{\alpha}{100}\right)^{\frac{T}{t}}
\]

(apoptotic cells appear every t h at a rate of \(\alpha\) percentage and cells are incubated for \(T\) h). Precise determination of \(t\), however, is a matter of debate. After cell fragmentation, apoptotic cells excrete apoptotic bodies and lose their cellular morphology within several h (22). Therefore, if it is assumed that \(t\) is 6 h, the growth rate of cells after 72 h exposure to 10 \(\mu\)M c-myc AS is simulated as

\[
\frac{1 - 3.5}{100} = 0.684
\]

(untreated and AS PS-ODN-treated cells revealed 0.4 and 3.5% apoptotic cells, respectively, according to the present study). This simulation fits very well with the actual data which showed that the number of the cells treated with 10 \(\mu\)M c-myc AS for 3 days was reduced to approximately 60% of the control growth. Therefore, it is likely that the inhibition of cell proliferation caused by continuous exposure to c-myc AS PS-ODN could be entirely explained by the constant induction of apoptosis during the culture. However, because this conclusion is based on the unproved assumption that the kinetics in AS PS-ODN treated and untreated cells is the same, further elucidations are needed.

Reduced c-myc protein levels by c-myc AS PS-ODN could be rapidly reversed within 1 h after its withdrawal. When HL-60 cells were treated with c-myc AS PS-ODN for 12 h and then cultured in AS-free medium plus 10% FCS, a pronounced increase in the number of apoptotic cells was observed. The increase in numbers of apoptotic cells preceded by up-regulation of c-myc expression after AS with-

Table 1
Analysis of cell cycle of HL-60 cells

<table>
<thead>
<tr>
<th></th>
<th>(G_1)</th>
<th>(S)</th>
<th>(G_{2-M})</th>
<th>Apoptotic cells^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>33^b (29)</td>
<td>53 (57)</td>
<td>13 (12)</td>
<td>0.8 (0.8)</td>
</tr>
<tr>
<td>S-treated</td>
<td>30 (27)</td>
<td>55 (56)</td>
<td>13 (14)</td>
<td>1.0 (1.0)</td>
</tr>
<tr>
<td>AS-treated</td>
<td>30 (29)</td>
<td>55 (55)</td>
<td>11 (13)</td>
<td>3.3 (3.0)</td>
</tr>
</tbody>
</table>

^a Cells in the hypodiploid and near triploid regions without incorporation of BrdUrd identified by flow cytometry.

^b Percentages of HL-60 cells in each cell cycle after treatment with c-myc AS PS-ODN for 3 days or 12 h (numbers in parentheses).

Fig. 6. Effects of c-myc AS PS-ODN on cell cycle. In A, cells in the hypodiploid and near triploid region without incorporation of BrdUrd could scarcely be found in untreated HL-60 cells. However, in B, such cells could be detected after exposure to c-myc AS PS-ODN for 12 h (arrows).
up-regulation of c-myc protein levels could also induce apoptosis in hallmark of apoptosis, was also observed. These findings indicate that withdrawal could be identified not only morphologically but also by constant effect on the induction of apoptosis and its abrupt up-regulation (6) or by the retroviral c-myc insertion (4, 5, 21) could induce appreciable apoptosis.

Although c-myc expression is likely to be necessary for activation-induced apoptosis, it is unknown whether c-myc expression alone is sufficient for the induction of apoptosis. Evan et al. (5) reported that cells with higher levels of c-myc protein are more prone to apoptosis upon serum deprivation. Askew et al. (21) also found that constitutive expression of c-myc is lethal to cells growing at high cell densities. In this study, however, serum level of the culture medium was always 10% and cell density was not so high.

We confirmed that both down- and up-regulation of c-myc expression could induce apoptosis of HL-60 cells in the present study and found that the continuous suppression of c-myc expression causes a constant effect on the induction of apoptosis and its abrupt up-regulation rapidly drives a considerable number of HL-60 cells to the apoptotic pathway. Thus the growth of leukemic cells may be potentially suppressed through manipulation of the induction of apoptosis by alterations of c-myc expression with c-myc AS PS-ODN.

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