Specific inhibition of the expression of the promyelocytic leukemia (PML) protein by anti-sense oligonucleotides

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Abstract
In the present study, using anti-sense oligonucleotides the inhibition of expression of the PML protein has been investigated. The anti-sense oligonucleotides were designed against the translation initiation site of the PML gene, and their effects were investigated on cellular growth and DNA synthesis. Incubation of normal human fibroblast cells with the anti-sense oligonucleotides resulted in the complete inhibition of the PML protein expression. Inhibition of the PML protein expression by anti-sense oligonucleotides was found to be associated with an increase in cellular growth and doubling time. Furthermore, in cells treated with the anti-sense oligonucleotides, but not sense or scrambled oligonucleotides (control), the cellular DNA synthesis also showed a marked increase, confirming the induction of cellular growth upon inhibition of PML synthesis. These findings clearly demonstrated that the inhibition of the expression of the PML protein could be achieved using the anti-sense oligonucleotides, providing a model for better investigation of the biologic role of PML in the cell.
Keywords: *Promyelocytic Leukemia; PML; Anti-sense oligonucleotides; Protein expression.*

INTRODUCTION

The promyelocytic leukemia, PML, is a nuclear protein (Borrow *et al*., 1990). It belongs to a family of proteins, which contain several functional domains, including the RING-finger, two cys/his rich regions designated the B box motifs and an a-helical domain, which is involved in PML/PML dimerisation (de The *et al*., 1991; Freemont *et al*., 1991; Kakizuka *et al*., 1991). In the nucleus, PML was found to be the major component of multiprotein nuclear complexes that have been variably termed Kremer bodies, ND10, PODs (for PML oncogenic domains), and PML-nuclear bodies (PML-NBs). These PML-NBs appear as foci within the nucleus when visualized with antibodies against PML or other NB-associated factors (Doucas and Evans, 1996). Many proteins co-localized with POD have been identified which involve in a broad spectrum of biological functions in the cell. These include SP100, the ubiquitin-like protein modifier SUMU-1/PIC-1/sentrin, the interferon induced protein ISG20, immediate early viral proteins IE1 and IE4 (Sternsdorf *et al*., 1997; Ahn and Hayward, 1997; Muller *et al*., 1998).

The promyelocytic leukemia (PML) protein functions as a cellular growth and transformation suppressor (Le *et al*., 1998; Liu *et al*., 1995; Mu *et al*., 1997). Recently, the protein has received considerable attention due its interaction with several cell cycle regulatory proteins including Sp1, p53, pRb and cyclic AMP binding protein (CBP) (LaMorte *et al*., 1998; Vallian, *et al*., 1998a; Alcalay *et al*., 1998). PML binds directly to p53 and recruits it to PML-NBs (Pearson and Pelicci, 2001). Current models suggest that recruitment to PML-NBs activate p53 by bringing it in close proximity with CBP/p300 (LaMorte *et al*., 1998). Acetylation of p53 by CBP/p300 then increases p53 DNA binding affinity, leading to an activation of p53-responsive genes. This activation of p53 likely contributes to the tumor suppressor function of PML (Le *et al*., 1998; Liu *et al*., 1995; Mu *et al*., 1997). In line

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with these findings, transient expression of PML induced G1 arrest and apoptosis in MCF-7 cells (Le *et al*., 1998). In HeLa cells, stable expression of PML resulted in growth inhibition by lengthening the G1 phase of the cell cycle (Mu *et al*., 1997). PML affects cell cycle progression by modulating the expression of several key proteins involved in G1/S checkpoint (Wang *et al*., 1998). However, the exact role of PML in cellular growth remains to be understood. In this study the aim was to examine the effects of anti-sense oligonucleotides on the expression of the PML protein and in cellular function.

MATERIALS AND METHODS

Anti-sense oligonucleotides: Anti-sense oligonucleotides used in this study were designed using the FOLDRNA software (Gaken *et al*., 1996). The oligonucleotides were selected against the translation initiation region of the PML gene. Two control oligonucleotides (sense and scrambled) were also designed. The sequence of the oligonucleotides is as follows:

Sense: 5´-GGTCCATGGAGCCTG-3´
Anti-sense: 5´-CAGGCTCCATGGACC-3´
Scrambled: 5´-GTACGATCGATCATG-3´

In order to increase the stability of the oligonucleotides in the culture medium as well as in the cell, the first four residues at the end of each oligonucleotide were chemically suphorized.

Cell culture and transfection: Normal human fibroblast cells were used. The cells were purchased from ATCC and cultured in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO/BRL, Gaithersburg, MD) in 5% CO_2 at 37°C in a humidified incubator. Since the cells can only be cultured for a limited generation, after first passage the cells were trypsinized and subcultured in 24 well dishes at 10^4 cell/ per well. In each dish, appropriate oligonucleotides at 50 or 100 pmol were added. After 24h the medium was refreshed by new medium containing similar concentration of oligonucleotides. The experiments were performed at least in triplicate.

Western blot analysis: The expression of PML protein in fibroblasts cells was evaluated using Western blotting as described previously (Wu *et al*., 2001). Briefly, after 48h incubation period, the cultured cells were washed and harvested in phosphate buffer saline (PBS) and lysed. Total cell extract was then prepared. Proteins in the samples were resolved on 10% SDS-polyacrylamide gel (SDS-PAGE), and then transferred onto nitrocellulose membrane. The membrane was probed with anti-PML antibody. Antibodies used were all polyclonal, which were prepared in rabbit. The anti-PML antibody was raised against a GST-PML fusion protein as reported (Vallian *et al*., 1998b).

Analysis of cell growth and DNA synthesis. The *in vitro* growth rate of fibroblast cells, which were incubated with oligonucleotides, was assessed using the trypan blue exclusion assay as described (Mu *et al*., 1997). For this assay, cells were plated at 1×10^4 cells /well in 24-well plates. The number of cells was determined at 24h intervals. The DNA synthesis rate of the cells which reflect cell proliferation was determined using the [^{3}H]thymidine incorporation assay. Cells were plated at 1×10^4 cells/plate in 24-well plates, and cultured in complete medium. Cells were then incubated with the oligonucleotides. [^{3}H]thymidine (2 mCi) was added to each well at 24, 48, and 72h, and the cells were incubated for additional 2h at 37°C. Cells were then washed in PBS and harvested as described (Mu *et al*., 1997). Radioactivity of the incorporated [^{3}H] thymidine was determined in a scintillation counter.

RESULTS

Inhibition of the expression of PML protein in the presence of anti-sense oligonucleotides: In order to analyze the effects of anti-sense oligonucleotides on the expression of PML protein, the oligonucleotides consist of anti-sense, sense and scrambled relative to the initiation of translation site of the PML gene were used. Anti-sense oligonucleotides were used at two concentrations, 50 and 100 pmol. As shown in figure 1, increase in the concentration of anti-sense oligonucleotides from 50 pmol to 100 pmol did not affect the inhibition of expression of PML. In contrast, the sense and/or scrambled oligonucleotides had no effect on the PML protein expression (Fig. 1, compare AS50 and AS100). These data clearly showed that the expression of the PML protein could be specifically inhibited by anti-sense oligonucleotides.

Specific inhibition of PML alters the cellular growth: Given the specific inhibition of the expression of PML protein by anti-sense oligonucleotides, and the fact that PML functions as a growth suppressor (see introduction), prompted us to examine the cellular growth following the inhubition of PML expres-

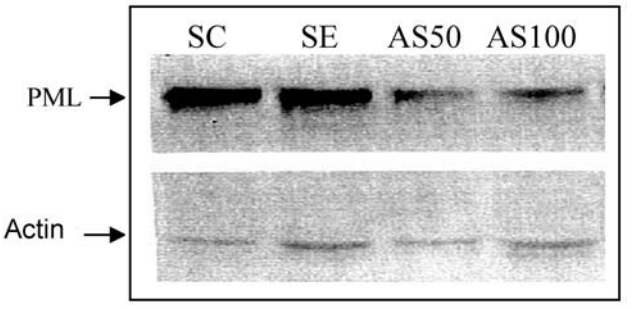

Figure 1. Analysis of expression of the PML protein in human fibroblasts. Expression of PML in the presence of anti-sense (AS), sense (SE) and scrambled (SC) oligonucleotides was analyzed. Anti-sense oligonucleotides were used at 50 (AS50) and 100 (AS100) pmol concentration as indicated. 48h after incubation, total cellular protein was extracted. Protein expression in each lane was normalized against the expression of actin. See Materials and Methods for more information.

sion. Therefore, the effects of anti-sense oligonucleotides (as above) were examined on cellular growth and propagation of the normal human fibroblast cells. The data as illustrated in figure 2, show that in the presence of antisense oligonucleotides the cellular growth was increased almost 2-fold. The cellular growth was monitored by counting the number of the colonies, which were produced by the cells in culture. However, in parallel experiments, the sense, scrambled and control (no oligonucleotides) showed no significant effects on the cell growth.

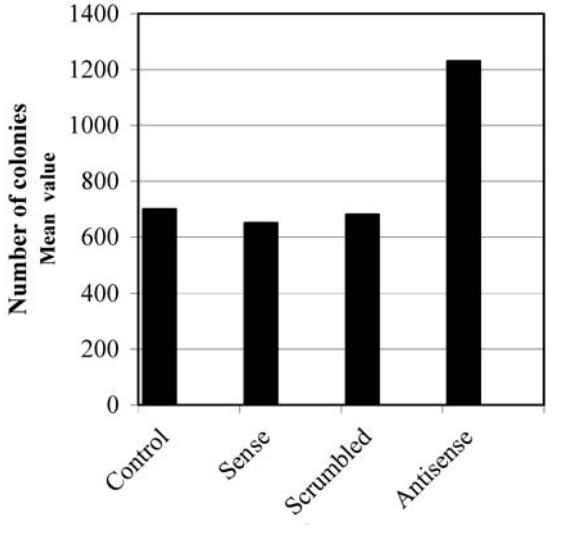

Figure 2. Analysis of cellular growth following treatment with oligonucleotides**.** The growth of the human fibroblast cells was examined 48h after incubation in the presence of anti-sense oligonucleotides as indicated. The mean values for the number of colonies were presented. Control represents the cells without treatment with oligonucleotides.

Increase in DNA synthesis rate following the inhibition of PML expression: Increase in cellular growth is not always in conjunction with DNA synthesis. Therefore, it was of great interest to investigate the effects of PML on DNA synthesis rate in the cells treated with anti-sense oligonucleotides. Therefore, the effects the the oligonucleotides (as in figure 1 and 2) were analysed on DNA synthesis. The synthesis of DNA was monitored by means of incorporation of labeled thymidine (see Matherials and Methods) following the treatment of the cells with the oligonucleotides. As shown in figure 3, in the presence of antisense oligonucleotides, there is a sharp increase in DNA synthesis nearly 24 after incubation (up to 2-3 fold). The synthesis rate of DNA was then decreased, which is followed by cell division. In contrast, the sense and scrumbled oligonucleotides showed almost no significant effects on DNA synthesis (Fig. 3).

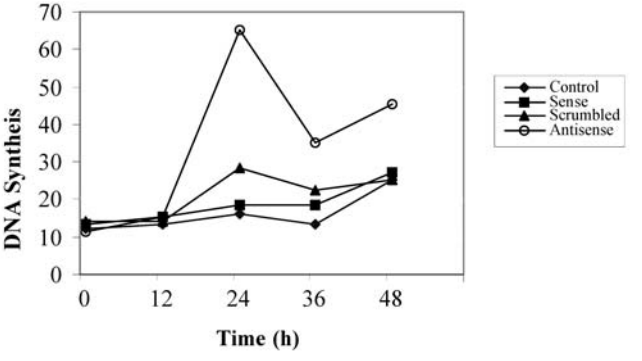

Figure 3. Assessment of DNA synthesis rate in the presence of oligonucleotides. The rate of DNA synthesis was examined using Thymidine incorporation assay as described in Materials and Methods. Control represents the extracts from cell without treatment with oligonucleotides. DNA synthesis was measured as count per minute (CPM) of the incorporated labeled Thymidine at the interval times, as indicated. The mean values from at least three independent experiments are represented.

DISCUSSION

In this study the effects of inhibition of PML protein expression was investigated on cellular growth. Previous studies showed that PML has a negative effect on cellular growth and transformation (Le *et al*., 1998; Liu *et al*., 1995; Mu *et al*., 1997). This supports the notion that the proper expression of the intact PML protein is required for normal cellular function. Interestingly, the PML gene was found to express in all the cells tested, suggesting that it may function as a housekeeping gene (Flenghi *et al*., 1995). Therefore, the removal of the PML protein from the cell by inhi-

bition of its expression could be of great value as a model for analysis of the exact role of PML in cellular function. The results of this study clearly showed that the expression of PML in the presence of specific anti-sense oligonucleotides was significantly inhibited. Increase in the concentration of anti-sense oligonucleotides did not affect the inhibition of expression of PML. This may suggest that there might be a limit for the action of anti-sense inhibition in the cell.

The cellular growth and propagation of the fibroblast cells were found to be increased in the presence of antisense oligonucleotides. However, the sense, scrambled and control (no oligonucleotides) had no significant effect on the cell growth. These results could demonstrate the necessity of the presence of PML for normal cell growth. The number of cells, which represents the doubling time was also increased. Increase in doubling time may be related to the known negative effects of PML on cell cycle progression (Xu *et al*., 2005; Le *et al*., 1998). As represented in figure 3, after a sharp increase at 24h, there is a decrease in DNA synthesis, which id followed by increase in the number of the cells (data not shown). Together the data support the previous reports on the negative effects of the over-expression of PML on cellular function (Mu *et al*., 1997). The reports on the biologic role of PML in the cell showed that this protein could also function as a negative regulator of transcription of several target genes (Vallian and Chang, 2004). It specifically suppresses the promoter activity of the epidermal growth factor receptor (EGFR) gene promoter (Vallian and Chang, 2004). It was found that this inhibitory effect was exerted through the SP-1 mediated activity of the EGFR promoter (Vallian *et a*l., 1998a). In the present study, the inhibition of expression of the PML protein in the presence of anti-sense oligonucleotides, resulted in the increase in cell growth and DNA synthesis (Fig. 3). Increase in DNA synthesis following the reduction of PML expression may indicate the importance of this protein for normal cellular function. Moreover, the data could suggest that the low level expression of the PML protein is necessary to keep the rate of the DNA synthesis in a controlled manner. This notion is supported by the findings that the PML protein was physically and functionally associated with the retinoblastoma (pRb) and p53 tumor suppressor proteins (Alcalay *et al*., 1998; Pearson and Pelicci, 2001). It is, therefore, could be further hypothesized that interaction of PML with the cell cycle regulatory protein was required for normal cell function. The elimination of PML could abrogate PML/Rb and/or PML/p53 function, resulting in increased DNA synthesis and cellular growth.

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