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Letter to the Editor

Prothymosin α antisense oligonucleotides induce apoptosis in HL-60 cells

Dear Editor,

Prothymosin α (ProTα) is a highly acidic protein widely distributed in mammalian tissues whose expression is directly related to cell proliferation. Thus, the levels of ProTα mRNA increase when fibroblasts and T-lymphocytes are stimulated to proliferate[1,2] as well as during liver regeneration.[3] Moreover, incubation of myeloma cells with antisense oligonucleotides complementary to the 5′ end of ProTα mRNA blocked their proliferation[4] and overexpression of ProTα accelerates proliferation, shortening the G1-phase of the cell cycle, and retards differentiation in HL-60 cells.[5] An important finding was that c-*myc*, one of the most prominent proto-oncogenes implicated in the control of cellular proliferation, directly induces transcription of the ProTα gene.[6] Recently, we have provided, for the first time, strong evidence about the function of ProTα in the remodelling of chromatin provoking the unfolding of chromatin fibres through its interaction with histone H1.[7]

Since ProTα expression is regulated during cell proliferation and differentiation,[8,9] we analyzed the effect that treatment with ProTα antisense oligonucleotides (PAS) would produce over these cellular processes using the human promyelocytic leukemia HL-60 cell line. HL-60 cells were cultured in the presence of antisense (5′-GTC-TACGGCTGCGTCTGACAT-3′) or sense (5′-ATGTCAGA-CGCAGCCGTAGAC-3′) oligonucleotides with base sequences complementary and identical, respectively, to those of ProTα mRNA.[4] Concentrations up to 40 μM of PAS provoked a slight inhibition of proliferation but when the concentration was increased to 80 μM, we found a remarkable inhibition (∼50%) of proliferation compared to the cells cultured in the absence of oligonucleotides or in the presence of the sense oligonucleotide (Figure 1a). This result agrees with a previous report[4] in which treatment of RPMI 8226 human myeloma cells with PAS inhibited their division.

The treatment of HL-60 cells with c-*myc* antisense oligonucleotides produces a decay in their proliferation rate as well as an induction of differentiation.[10] Since c-*myc* acts as a transcriptional regulator of ProTα and both genes are expressed more actively during the cell proliferation descending their mRNA levels during the course of differentiation, it is possible that treatment of HL-60 cells with PAS could also induce their differentiation. However none of the concentrations used (up to 80 μM) provoked the differentiation of HL-60 cells perhaps because such concentration was not enough to produce a strong depletion of the intracellular levels of ProTα mRNA. To verify this possibility, we treated the HL-60 cells with 160 μM of PAS. We found a very strong inhibition of the proliferation rate. Moreover, there was an obvious influence of such PAS concentration over the cell viability since after 3 days of culture many cells appeared to be dead. (Figure 1). This mortality was not due to exogenous effects since identical concentration of sense oligomer did not affect either to proliferation or cell viability.

To find out if the mortality was due to the triggering of apoptosis, we first carried out a flow cytometric analysis. As shown in Figure 1C, the HL-60 cells treated with 160 μM of PAS showed a hypodiploid DNA peak clearly discernible from the diploid DNA peak; this peak is the result of the reduced DNA content characteristic of apoptotic cells. The percentage of apoptotic cells was ≈23%. In contrast, cells treated with an equivalent amount of ProTα sense oligomer as well as untreated HL-60 cells did not show the hypodiploid DNA peak. The biochemical hallmark of apoptosis is the degradation of genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). To analyze the genomic DNA, aliquots of HL-60 cells were incubated with ProTα sense or antisense oligonucleotides (160 μM). After 3 days of incubation, their DNAs were extracted and analyzed by electrophoresis. As shown in Figure 1B, the DNAs prepared from HL-60 cells cultured in the absence of oligonucleotides or treated with the ProTα sense oligomer, were not fragmented showing a high molecular weight. In contrast, in the HL-60 cells treated with PAS, some of the genomic DNA appeared fragmented generating a ladder composed by DNA bands multiples of 180–200 bp, a distinctive pattern of apoptotic cells. Therefore the presence of a high concentration of PAS is provoking the cell death by apoptosis probably preventing or inhibiting the translation of ProTα mRNA.

Apoptosis is an evolutionarily conserved mechanism of programmed cell death present in eukaryotic cells. Several lines of evidence strongly indicate a relationship between apoptosis and cell proliferation and, in fact, many oncogenes that control cell cycle progression can also induce apoptosis.[11] The nuclear Myc protein is a potent inducer of both proliferation and apoptosis and up-regulates the expression of ProTα. Interestingly, incubation with c-*myc* antisense oligonucleotides enhance the induction of apoptosis in HL-60 cells[12] and in this work we have observed a similar induction in those cells using PAS. Is there any link between both

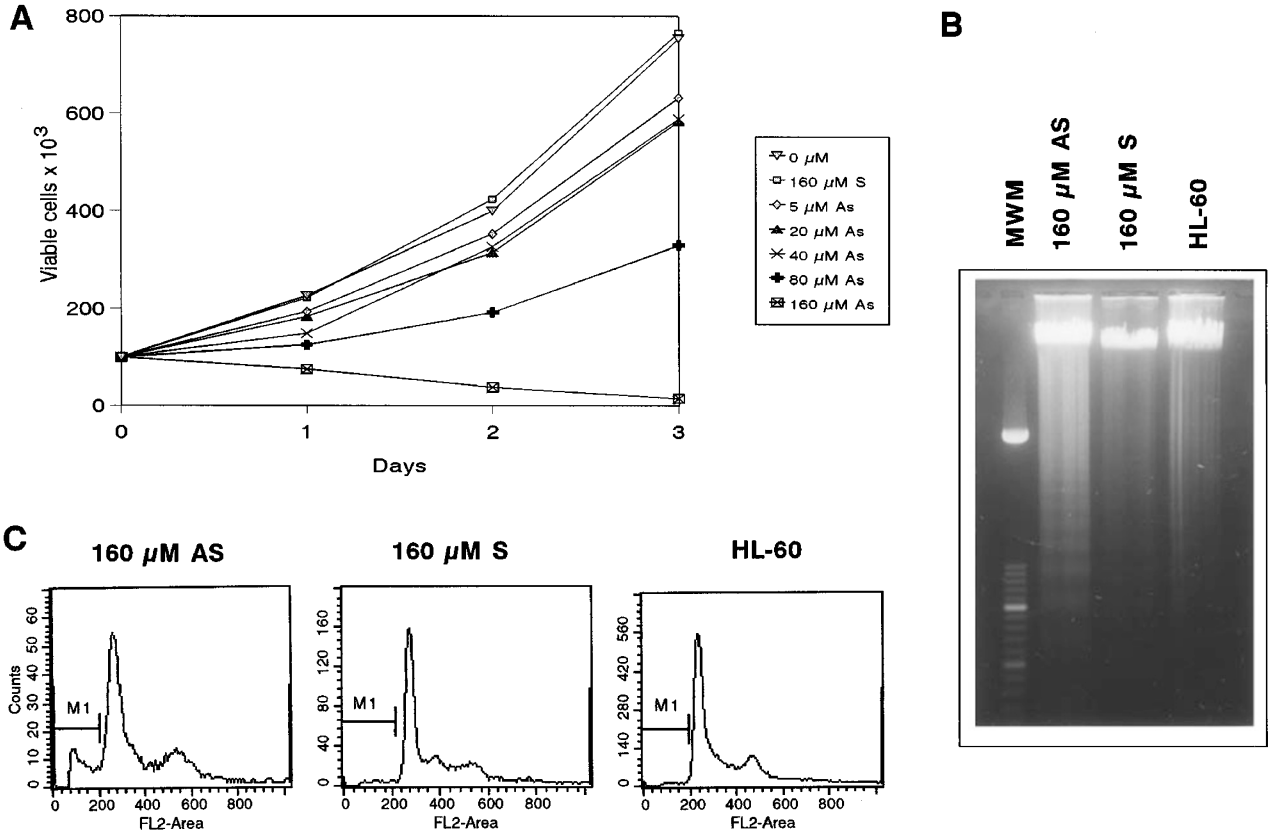

Figure 1 Effect of ProTα antisense oligonucleotides on proliferation and apoptosis. (**A**) Dose-dependent inhibition of the HL-60 cell proliferation by PAS in suspension cultures. Triplicate aliquots of exponentially growing HL-60 cells (100 000) were resuspended in 200 μl of culture medium and different concentrations (inset in **A**) of sense (control) or antisense oligonucleotides were added. After an incubation of 1 h with the oligonucleotides in the absence of serum, to avoid the action of nucleases, cells were incubated for 3 days with 10% serum, determining their number and viability every 24 h. Data are presented as mean values from triplicate cultures. (**B**) Analysis of DNA fragmentation. For genomic DNA extraction, HL-60 cells were harvested, washed and incubated in lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and RNase A [20 μg/ml]) for 1 h at 37°C. After addition of proteinase K (100 μg/ml), the samples were incubated at 37°C for 4 h, extracted with phenol:chloroform, precipitated with one volume of isopropanol and finally resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. DNA samples were separated on 2% agarose gel. MWM represents the DNA Molecular Weight Marker (a 50 bp ladder) from Boehringer Mannheim. (**C**) Flow cytometric analysis. DNA fluorescence histograms of propidium iodide-stained HL-60 cells without treatment (HL-60) and treated with 160 μM of ProTα sense oligonucleotides (S) or with the same concentration of antisense oligonucleotides (AS). After culturing the cells in the presence of 160 μM antisense or sense ProTα oligonucleotides, cells were harvested, washed in PBS, fixed in 1% paraformaldehyde, permeabilized with 96% ethanol, RNase treated and stained with propidium iodide (20 μg/ml). FACS analysis was carried out in a FACSCALIBUR cytometer (Becton-Dickinson) and the data were analyzed using a software for DNA analysis (Verity Software House). Apoptotic cells are indicated by the M1 bar

findings? There are several possibilities to explain the induction of apoptosis by c-*myc* although most of them implicate transcriptional mechanisms.[11] Since c-*myc* inhibition provokes a decrease in the levels of ProTα, it is reasonable to speculate that in the induction of apoptosis mediated by c-*myc* an essential point would be the inhibition of the ProTα gene expression. An implication of the present results is that the growth of leukaemic cells might be potentially suppressed through manipulation of the induction of apoptosis and inhibition of cell proliferation by alterations of c-*myc* and/or ProTα expression with antisense oligonucleotides. The elucidation of the molecular links between c-*myc* and ProTα in the induction of apoptosis may be crucial in the understanding of cancer biology.

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