Antiproliferative Effect of DNA Polymerase α Antisense Oligodeoxynucleotides on Breast Cancer Cells

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INTRODUCTION

The two major events in the cell cycle are DNA replication and cell division. Regulation of cell growth, as the switching mechanism between quiescence and proliferation, is defective in cancer cells. A key component of chromosome replication in eukaryotic cells is DNA polymerase α (polα), which plays a fundamental role during the proliferative cycle. This enzyme consists of a family of polypeptides with catalytic activity ranging in size from 125 to 180 kDa, a 70-kDa subunit of unknown function, and two polypeptides of 49 and 55 kDa associated with the DNA primase activity [1]. The polα activity sharply increases during progression from the G0 to S phases of the cell cycle. Furthermore, its level is highest in rapidly growing tissues, cultured tumor cells, and mitogen-stimulated lymphocytes. Within neoplastic cells, significant amplification of polα mRNA contents, as well as increased enzymatic activity, have been reported [2, 3].

Since the expression of polα gene is closely coupled to the onset of DNA replication, elevated in transformed cells, and down-regulated in terminally differentiated cells, polα may represent a potential target for the genic control of neoplastic growth. Conventional antitumor drugs exert their mechanism of action by exploiting biochemical and kinetic differences between malignant cells and their normal counterparts, lacking in specificity. Specific inhibition of the synthesis of altered or overexpressed proteins, relevant for tumor growth through the control of their gene expression, might prove suitable as an alternative approach to experimental therapy of tumors. In particular, the antisense oligonucleotides might provide a rational methodology for the development of a new generation of potential antitumor compounds endowed with high therapeutic activity [4].

Antisense oligodeoxynucleotides (asODNs) are short synthetic nucleotide sequences complementary to RNA or DNA which can be used to block the expression of specific gene by annealing to complementary nucleic acid and affecting the transcription or the translation of the message [5]. Recent studies reported strong evidence that the expression of cell cycle-related genes might be negatively regulated by asODN, leading to the arrest of cell growth [6–9]. To this purpose, ODNs complementary to the translation start site of the polα
mRNA have been synthesized. Here we report in vitro evidence that antisense ODNs to pola are able to inhibit the growth of human cancer cells.

MATERIALS AND METHODS

Cell lines and treatment conditions. MDA-MB 231 and MCF-7 (human breast adenocarcinoma), SW 626 (human ovarian adenocarcinoma), DHL 4 (human B lymphoma), Jurkat (human acute T cell leukemia), and K-562 (human chronic myelogenous leukemia) cell lines were used in the present study. Cells were maintained at 37°C and 5% CO₂ in RPMI 1640 (Gibco) supplemented with penicillin, streptomycin, glutamine, and 10% (v/v) fetal calf serum (Gibco) previously checked for low nuclease activity. Cultures were plated in 35-mm petri dishes (3 x 10⁵ cells/dish), treated with sense and antisense oligomers every 24 h, and counted, daily, by the trypan blue dye exclusion method.

Synthesis and purification of oligomers. Unmodified 18-mer oligomers were prepared by means of an automatic synthesizer (Beckman) and purified by high-pressure liquid chromatography on a C18 column. Four ODNs were synthesized: two targeted to the translation start site and two directed to open reading frame regions of the pola cDNA sequence. The most effective ODN, containing the ATG initiation codon from nucleotide -3 to +15 of pola cDNA [1], was selected for this study. The sequences of these oligomers were: sense 5'-d(AACATGGCACCCGTGCAc) (sODN) and antisense 5'-d(GTGACACGGTGCATG) (aODN).

MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric method which measures cell growth and survival; tetrazolium salts are reduced to a blue-colored product (formazan) by intracellular dehydrogenases of viable cells. The amount of formazan, directly proportional to the number of cells, can be measured spectrophotometrically. Cells were seeded in 96-well plates (5 x 10⁵ cells/well) and treated, after medium change, with sense and antisense ODNs at the concentration of 10 μM, every 24 h. MTT assay was carried out daily by removing the medium from cultures and adding 50 μl of MTT solution (2 mg/ml) to each well. Following incubation (4 h at 37°C by shaking), 150 μl of DMSO was added to dissolve formazan crystals and plates were read on a Titertek Multiscan spectrophotometer (540 nm) [10].

[³H]Thymidine incorporation. Tumor cells were plated on four-well tissue culture chamber slides (Labtek, Nunc) at the initial concentration of 2 x 10⁴ cells/well. Antisense and sense oligomers (10 μM) were added in fresh medium at the time of plating and every 24 h. Cells were incubated with [methyl-³H]thymidine 2 μCi/well (87 Ci/mmol; Amersham) following the procedure previously described [11].

Flow cytometry. Cells (3 x 10⁶/plate) were cultured in 35-mm petri dishes (Nunc) and exposed to antisense and sense ODNs (10 μM) every 24 h. After harvesting cells were washed once in cold PBS one time, fixed in 1 ml of 95% ethanol, and stored at 4°C. Before analysis, cells were resuspended in PBS in the presence of propidium iodide (10 μg/ml), Tween 20 (0.005%), and ribonuclease (50 μg/ml) (Sigma) for DNA staining [12]. Flow cytometry was performed on an EPICS Elite cytofluorimeter (Coulter). DNA analyses were carried out using the "Multicycle" software on a Intel 386 PC.

Immunoprecipitation and SDS-PAGE. Cells (1 x 10⁷/plate) were plated in 60-mm petri dishes and treated with sense and antisense ODN (10 μM). After 24 h of incubation the medium was replaced by RPMI (methionine-free) (ICN Flow) without FCS supplemented with ODNs and cells were metabolically labeled with 50 μCi/ml [³⁵S]methionine (1129 Ci/mmol; DuPont–NEN) for 4 h. Labelled cells were collected by scraping and resuspended in 0.1 ml of lysis buffer [13].

Cell extracts were immunoprecipitated by monoclonal antibodies (MoAb) to pola recovered from the supernatant of SJK 132-20 hybridoma cell line (American Type Culture Collection) and with normal mouse antisera (Sigma) as control of MoAb specificity. Immunoprecipitation was carried out according to the procedure previously described by Thommes et al. [13].

Immunoprecipitated proteins, resuspended in 50 μl of denaturing sample buffer, were separated on a 7.5% SDS–polyacrylamide gel. The gels were then dried and exposed to film (X-Omat, Kodak) for 5 days at -80°C with a Cronex Lightning Screen; single-dimension scanning of the resulting film was analyzed by LKB Ultrascan XL laser densitometer.

RNA analysis. Total RNA was extracted from frozen cell pellets (1 x 10⁷ cells) by homogenization in 4 ml of 4.23 M guanidine thiocyanate (Fluka), 0.5% N-lauroyl sarcosine, 25 mM sodium citrate, 0.1 M β-ΜΕ, 5 μg/ml Escherichia coli RNA, and 0.3% anti-fade (Sigma). The homogenization buffer was adjusted to pH 7.3. The homogenate was loaded on a CsCl (ICN) gradient in a SW 41 polyallomer tube and centrifuged as described [14]. Fifteen micrograms of RNA were size fractionated on 1% agarose gel in 0.2 M formamide, blotted to Gene Screen Plus membrane (NEON), and uv crosslinked [14]. Forty nanograms of HindIII–BamHI 7.1-kb fragment of pCDKβ-pola vec were 2³P-labeled by the random-priming method [15] and utilized for the Northern blotting hybridization (10³ cpn/mg). The plasmid pCDKβ-pola was kindly provided by Dr. T. S. F. Wang.

Hybridization and washes were performed as reported by Biassoni et al. [14]. Northern blots were exposed to X-ray film (X-Omat, Kodak) at -80°C. Quantitative densitometric scanning of autoradiographs was performed by LKB Ultrascan XL laser densitometer.

RESULTS

The human breast cancer cells, MDA-MB 231, were exposed to an optimal dose (10 μM) of oligomers previously selected on the basis of dose–response curves [16]. Although this ODN concentration was able to induce a similar pattern of inhibition in different cell lines, as in Table 1, the present study more extensively reports the biological and molecular findings obtained with the MDA-MB 231 cells. Cultures were treated with oligomers every 24 h for four consecutive days.

Higher antiproliferative effects were observed after 2 days of exposure to sODN and asODN with 15 and 50% of inhibition vs untreated controls, respectively, as evaluated by MTT assay (Fig. 1). Furthermore, a concomitant reduction in the number of viable cells was also shown as in Fig. 2. Since pola is directly involved with DNA synthesis, the effects of asODN on the proliferat-
tive activity and cell cycle were studied by [³H]-thymidine incorporation and flow cytometry.

As reported in Fig. 3, the percentage of cells in DNA synthesis was highly reduced following exposure to asODN, reaching 90% of inhibition at 24 h, while similar effects did not occur with sense-treated cells. This significant decrease of labeled cells was presumably associated with the arrest at the G1/S border of the cell cycle. By flow cytometry, in fact, it was shown that 64% of antisense-treated cells were accumulated in the early S phase and 18% were in the G1 phase, whereas less than 1% of cells were in G2-M, at 24 h. (Fig. 4). Moreover, the presence of a hypodiploid peak (18% of cells/total) in the "antisense" DNA histogram was also observed. Further addition of antisense oligomer, however, no longer affected the cell growth rate or the G1/S block, beyond 24-48 h.

In order to assess whether the antisense might interfere with polα mRNA, equal amounts of total RNA (verified by ethidium bromide staining) from treated and control cultures, were used for northern hybridization. A 50% reduction in the 5.8-kb signal was detected in the MDA-MB 231 cells following 24 h exposure to asODN (Fig. 5). In addition, the synthesis of DNA polymerase α polypeptides was analyzed by SDS-PAGE of the immunoprecipitated MDA-MB 231 lysates with the monoclonal antibody SJK 132-20 to polα catalytic subunit. As shown in Fig. 6 the incubation of cells for 24 h with 10 μM asODN markedly reduced (39%) the intensity of the 180-kDa band compared to control and sense-treated cells.

DISCUSSION

The role and activity of DNA polymerase α has been widely covered in numerous articles and reviews during the last few decades [3, 17-19]. However, despite conflicting data on its intracellular amount and distribution, polα definitely plays a pivotal role in DNA replication and represents a primary target for antiproliferative agents. Since known antitumoral drugs mainly act by exploiting quantitative more than qualitative differences between tumor and normal cells, the genetic control of specific genes involved in the process of cancer transformation is largely pursued. To this end, the antisense strategy would appear to offer several advantages over cytotoxic agents for the management of neoplastic growth [20].

The results presented here demonstrate that the 18 mer complementary to the translation start site codons of polα exerts inhibition of the proliferative rate of MDA-MB 231 breast cancer cell line, while the corresponding sense oligomer failed to show significant inhibitory effect. It should be emphasized that the concentration of our unmodified oligomer is generally lower than those reported in other studies also employing oligonucleotide sequences.
ANTISENSE OLIGOMERS INHIBIT MDA-MB 231 GROWTH

FIG. 5. (a) mRNA levels of human DNA polymerase α in MDA-MB 231 cells. Lanes: 1, control; 2, sense; 3, antisense. Equal amounts of total RNA were used for Northern blot analysis and verified by ethidium bromide staining of rRNA bands (b). The positions of 18S and 28S ribosomal RNA (left) and 5.8-kb pol α mRNA (right) are indicated.

FIG. 4. DNA content histograms of MDA-MB 231 cells during 24 h incubation with 10 μM oligomers. (A) control, (B) sense, (C) antisense. DNA contents of individual cells were determined by measuring the red fluorescence of propidium iodide-stained cells on a computer-operated cytofluorimeter (---, experimental results; —, multicycle DNA analysis; shaded area corresponds to the S phase; Ap, apoptotic cells with hypodiploid DNA content).

cleotides analogues [8, 21]. Cells exposed to asODN present a markedly decreased DNA synthesis (90% of inhibition) and accumulate in the G1/S phases of the cell cycle at 24 h, as revealed by [3H]thymidine incorporation and flow cytometry, respectively. Supposedly, in the presence of the asODN, cells that have entered S phase can no longer synthesize DNA, whereas those in other phases proceed along the cell cycle and stop at G1/S border. Moreover, the hypodiploid peak, observed in the DNA histogram of antisense-treated cells, might indicate the presence of 18% of apoptotic cells, as sug-

FIG. 6. Effects of oligomers on pol α protein synthesis. SDS-PAGE analysis of immunoprecipitated [35S]methionine-labeled MDA-MB 231 cell lysates by MoAb (SIK 132/20). Lanes: 1, control; 2, antisense; 3, sense. Molecular size markers and 180-kDa pol α peptide are indicated at right and left, respectively.
state transcript [23]. In addition its synthesis does not depend on the level of active polymerase since increased enzymatic activity is largely caused by phosphorylation-dependent regulatory process on preexisting pole [1, 23].

It has been claimed in several reviews that antisense ODNs could act at the transcriptional or post-transcriptional levels by binding directly to DNA or mRNA [5]. Present results, from Northern analysis and immunoprecipitation assay, showed a 50% reduction in the 5.8-kb signal of the antisense-treated cells as well as a 39% decrease in the amount of the 180-kDa polypeptide, suggesting post-transcriptional mechanisms of action. These probably involve the formation of an asODN/RNA hybrid which might impair the function of the mRNA in different ways, such as: (a) the degradation of RNA by RNase H activity, (b) the blockage of the mRNA transport from nucleus to cytoplasm and (c) the inhibition of mRNA translation into protein.

In the current study, the reduction of polα protein expression, correlated with a decreased of polα mRNA, supports the hypothesis of a degradation of the mRNA/antisense duplex by RNase H, in agreement with other previous reports [24–26].

In conclusion, the antiproliferative effects of the antisense oligomer to polα are demonstrated and additional prospects for the control of genes critical to cell growth are provided.

We thank Dr. Teresa S-F. Wang (Dept. of Pathology, Stanford University School of Medicine, Stanford, CA) for providing the plasmid pcDKBpoler and Dr. G. Melioli (Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) for the flow cytometric analyses. This research was supported by Associazione Italiana per la Ricerca sul Cancro and Consiglio Nazionale delle Ricerche grants.

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