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Oligonucleotide degradation contributes to resistance to antisense compounds

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A subline of the human B cell lymphoma DHL-4, grown in the artificial serum-free medium HB101, displayed a resistant phenotype to the activity of an antisense oligodeoxynucleotide (aODN) effective on the parental DHL-4 line. It was found that the cellular uptake of the 18mer aODN in the two cell lines was almost the same. In contrast, the unresponsive subline DHL-4r degraded the aODN very efficiently, in contrast to the stability of aODN inside cells of the parental DHL-4 line. Activation of the degrading 'machinery' combined with selective properties of the artificial medium may be responsible for the loss of responsiveness to aODN.

Key words: Antisense oligodeoxynucleotides, cellular uptake, resistant cells, stability.

Introduction

Antisense oligodeoxynucleotides (aODN) have emerged as fruitful ways to study gene expression[1,2] as well as potential therapeutic agents.[3–5]

However, activity has been viable in different cell sublines from the same genetic background and differentiation pathway.[6,7] There is very little information available about cell sensitivity after long-term treatment with aODN compounds. Studies relative to the biochemical mechanisms likely to be responsible for either reduced activity or loss of activity are at present marginal and have only recently been considered worth attention.[8,9] Natural or induced resistance might play important roles in the planning of effective aODN therapy for a number of diseases, including cancer and viral infections.

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In a program aimed to exploit antisense compounds to study genomic alterations at molecular level associated with neoplastic transformation, chromosome translocations have been targetted for antitumor activity. An 18mer oligonucleotide inhibited the *in vitro* growth of a human B cell lymphoma carrying the translocation t(14; 18).[10]

In the present study, lack of responsiveness to the effective aODN, designed to encompass the junction region of the hybrid gene *bcl2*–IgH carried by the DHL-4 human B cell lymphoma,[11,12] was shown by a DHL-4 subline. The cellular uptake and degradation of aODN was studied in the operatively resistant subline, originated by long-term growth in serum-free artificial medium, hereafter designated DHL-4r.

Materials and methods

Cell culture

Parental DHL-4 cells, obtained from Dr D Delia (NCI, Milan, Italy), were grown in RPMI 1640 containing 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere saturated with water and maintained in the logarithmic phase with greater than 90% viability as controlled by the Trypan blue dye exclusion test. The DHL-4r subline was obtained from the DHL-4 line after 3 months of growth under the same conditions in the serum-free medium HB101 (Irvine Scientific, CA USA).

Oligodeoxynucleotide

Purchased from Primm (Milan, Italy), the 18mer oligodeoxynucleotide (aODN) pGGTCCGAGGT-GGACTACT (pN18) was labelled with 5′-[^{32}P]ATP

(5000 Ci/mmol; Amity PG, Milan, Italy) using T4 polynucleotide kinase and purified by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions, followed by chromatography on a Sep-Pak C_{18} column and by precipitation with ethanol, following the method described elsewhere.[14]

Uptake assay

Parental or resistant cells, 1.5×10^5 ml, plated in a 24-well plate were incubated at 37°C in serum-free HB101 medium containing 1 μM of the 5'[^{32}P]pN18. At different times the cells were pelletted and 10 μM of supernatant frozen immediately.

Total radioactivity in three washes with the medium and in the cell pellets was counted as described.[15] The cellular uptake was expressed as the amount of aODN/10^6 cells.

Stability assays

Supernatants, obtained as described after 30 minutes to 24 h by adding 1 μM of [^{32}P]pN18 to the serum-free medium incubated either with 10^6/ml DHL-4 cels or with 10^5/ml DHL-4r cells, were run in denaturing 20% PAGE. Degradation of radiolabelled aODN was quantified by scanning the photographic negatives of gels with a densitometer and the area under the scan integrated.

Cells (10^6/ml) in HB101 medium were incubated for 2 h with 1 μM [^{32}P]pN18, washed and again cultured for 30 minutes to 22 h in fresh medium without [^{32}P]pN18. At the desired times, the cells were obtained and the supernatants lyophilized. Cells were lysed with 2% SDS, 1% β-mercaptoethanol and 7 M urea. Polynucleotides were extracted with phenol–chloroform[16] and [^{32}P]pN18 stability analyzed by PAGE as above.

To investigate degradation of aODN released by the cells, the lyophilized supernatants were redissolved, precipitated with 2% $LiClO_4$ in acetone and analyzed by PAGE as above.

Results and discussion

The growth of the human DHL-4 B cell lymphoma is inhibited in a very specific fashion by the aODN pN18 used here. It complements the unique nucleotide sequence originating from the junction

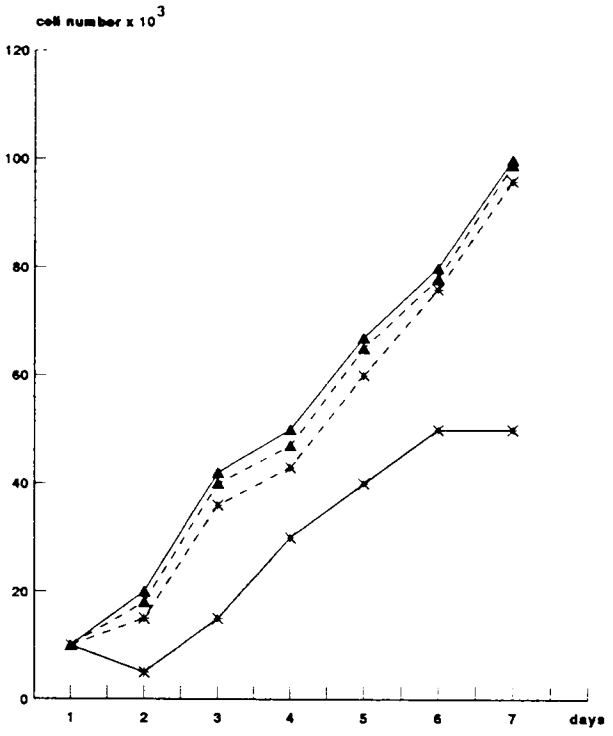

Figure 1. Time course of growth of DHL-4 cells and DHL-4r cells treated with aODN 10 μM. *, DHL-4; ▲, DHL-4r; ---, S; ——, AS.

region of the hybrid gene *bcl2*–IgH. The chromosomal translocation t(14;18) from which the chimeric gene derives might contribute to B cell transformation. It has been found that the growth of the DHL-4 cell subline (DHL-4r), obtained after 3 months of passage in the artificial medium HB101, was not inhibited by the aODN that does inhibit the growth of the parental DHL-4 cultured in RPMI 1640 containing 10% FCS (Figure 1).

Studies at the cellular level were done to compare the pharmacokinetic behavior of the aODN added either to the sensitive cells (DHL-4) or to the insensitive, hereafter resistant DHL-4r subline. As shown in Figure 2, the cellular uptake of the ^{32}P-labelled pN18 did not differ significantly in the two cell lines. However, when the [^{32}P]pN18 was removed after 2 h and incubation continued in the [^{32}P]pN18-free medium, there was a greater release of radioactivity from the DHL-4r cell samples (Figure 3). Although the smallest amount of radioactivity within DHL-4r cells was observed after 8 h of incubation, the radioactivity started to increase after this period, to the amount found within the parental DHL-4 cells at the end of 24 h of incubation. It might be hypothesized that

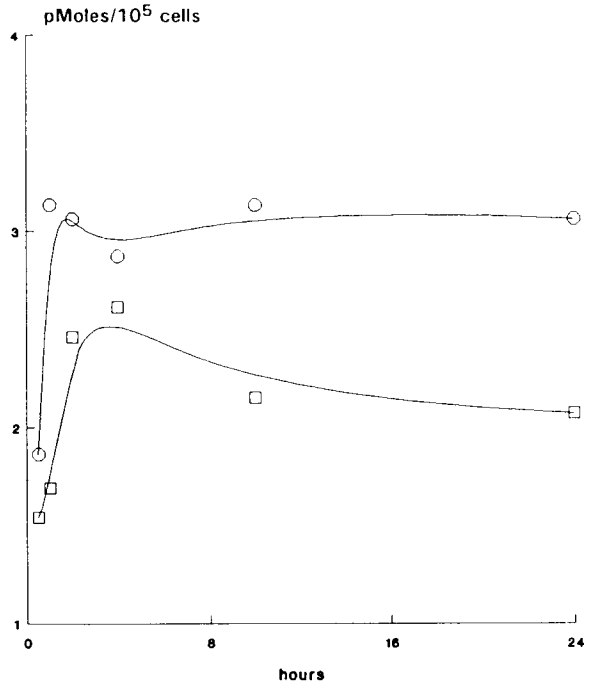

Figure 2. Uptake of [^{32}P]pN18, 1 μM, by DHL-4 cells in serum-free HB101 medium at 37°C, 10^5 cells/ml. □, DHL-4; ○, DHL-4r.

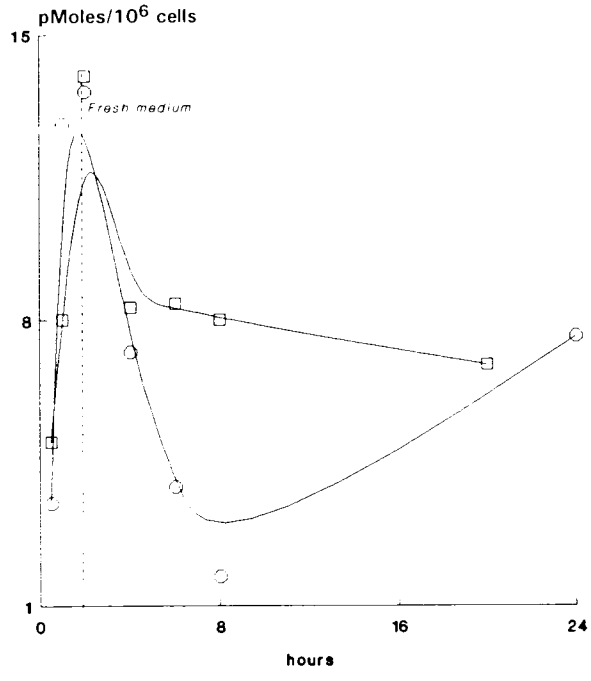

Figure 3. Uptake and release of [^{32}P]pN18, 1 μM, by DHL-4 cells in serum-free HB101 medium, 10^5 cells/ml. After 2 h, [^{32}P]pN18 was washed out, fresh medium added and incubation continued for 22 h. □, DHL-4; ○, DHL-4r.

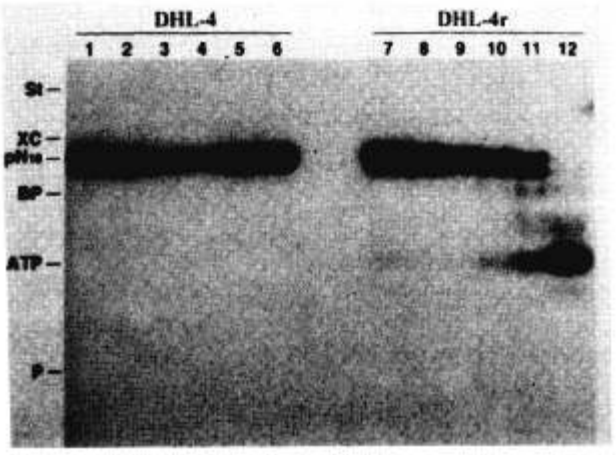

Figure 4. Degradation products of [^{32}P]pN18 in the DHL-4 or DHL-4r culture medium. Supernatants, DHL-4 lanes 1–6 and DHL-4r lanes 7–12, corresponding to 0.5, 1, 2, 4, 8 and 24 h of incubation, were analyzed by 20% PAGE. St, XC, BP, ATP, P, pN18: position of start and markers (xylene cyanole, bromphenol blue, radiolabelled ATP, phosphate and intact pN18).

radioactive phosphorus or low molecular weight oligos, probably produced by degradation of the original [^{32}P]pN18, might have been re-incorporated by the DHL-4r cells.

Degradation of oligonucleotide by the two DHL-4 lines was studied by PAGE. It was found, as shown in Figure 4 and in the data obtained by quantitation of scans of the photographic negatives of gels (not reported), that there are very different pathways of degradation. The [^{32}P]pN18 incubated with the DHL-4r cells underwent a time-dependent degradation that was complete after 24 h. In contrast, there was no obvious evidence of loss of aODN integrity in the culture medium of the parental, sensitive DHL-4 cell line (only about 10%).

Finally, the intracellular catabolism of [^{32}P]pN18 was investigated. Most of the [^{32}P]pN18 (85–90%) was found unchanged inside DHL-4 cells even after 24 h of incubation (Figure 5, lines 1–7). In keeping with the degradation of [^{32}P]pN18 in the supernatant, there was massive degradation of the oligonucleotide inside the DHL-4r cells. After 0.5 h of incubation, some 65% of the aODN had been degraded inside the DHL-4r cells to short oligonucleotides and phosphate. About 3% of ^{32}P label was incorporated in biopolymers. The data from Figure 5 and from scanning of negative gels show that short oligonucleotides and phosphate did not accumulate inside cells nor remain there during the 24 h. Phosphate was incorporated into

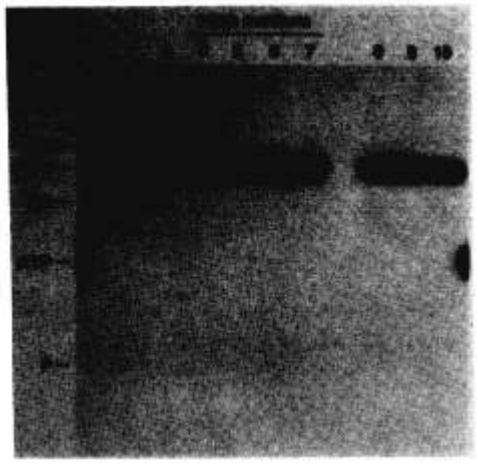

Figure 5. Stability of [^{32}P]pN18 inside DHL-4 cells. DHL-4 cells were incubated in HB101 medium with 1 μM [^{32}P]pN18 for 2 h, washed and further incubated for 22 h in [^{32}P]pN18 free medium. Lanes 1–7: PAGE analysis of radioactivity in the cell extracts at 0.5, 1, 2, 3, 4, 8 and 24 h of incubation. Lanes 8–10: PAGE analysis of radioactivity released in the supernatants after 4, 8 and 24 h of incubation.

biopolymers (4 h, about 11.5%) and released from the cells as soon as the short oligonucleotides and may be found in the medium (Figure 5, lines 8–10).

A few mechanisms have been hypothesized to explain the different degrees of sensitivity to aODN in cell sublines of the same genetic lineage. We provide here experimental evidence that activation of a degrading 'machinery' might be the reason for the loss of biological activity of aODN that are highly inhibitory to parental cells. It might be worthwhile to study the molecular mechanisms of aODN degradation and how they are activated. Since DHL-4 cells were originated by culture in an artificial medium without addition of oligonucleotide compounds, genetic instability of DHL-4 cells combined with the potentially selective properties of the medium might be the most obvious hypothesis to investigate first.

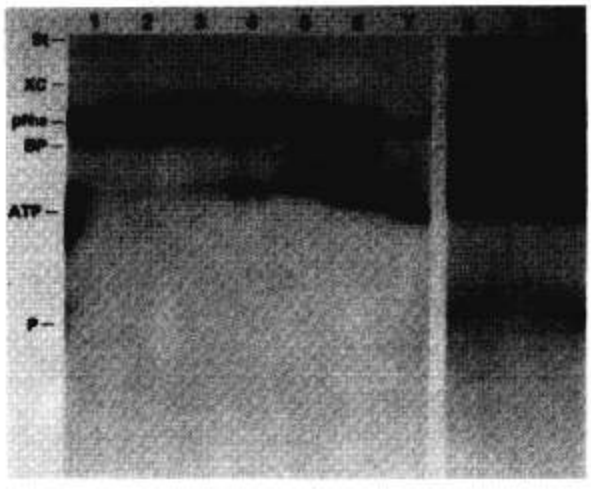

Figure 6. Stability of [^{32}P]pN18 inside DHL-4r cells. Conditions and lanes as Fig. 5.

Acknowledgments

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