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ANTISENSE OLIGODEOXYNUCLEOTIDES FOR THE TREATMENT OF CHRONIC MYELOGENOUS LEUKEMIA: ARE THEY STILL A PROMISE?

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Nucleic acid-based therapy holds the potential for a new era in the treatment of life-threatening disease. Advances in the use of antisense oligodeoxynucleotides (ODNs) as inhibitor of aberrant gene expression in neoplastic cells have been reviewed in a recent issue of this Journal;[1,2] since new reports are continuously appearing, the present state of the art needs to be defined again.

The optimism generated by promising results obtained in different cellular systems is tempered by the difficulties in developing clinical trials, mainly because of the lack of sufficient knowledge regarding the mechanisms associated with the inhibition of gene expression *in vitro* and *in vivo*. For example, the duration of ODN survival inside the cells, the most appropriate ODN length to produce specific inhibition of gene expression, ODN plasma levels, tissue distribution and concentration in animals are some of the aspects that need to be investigated and clarified before a drug can be developed for clinical use.

Antisense oligodeoxynucleotides for chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) represents a suitable candidate for antisense therapy. The reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11) generates the Philadelphia chromosome and the fusion gene BCR-ABL, which is transcribed into a mRNA and translated into a p210 BCR-ABL protein.[3,4] It has been clearly demonstrated that this gene and its product play a role in the pathogenesis of CML[5] and that the greater intrinsic protein tyrosine kinase activity of p210 is presumably responsible for its transforming potential.[6] The bcr-abl hybrid transcripts generated by the t(9;22) translocation are ideally suited for antisense-based therapy; furthermore, CML remains a fatal disease which might benefit from experimental approaches.

Two genes, *c-myb* and *bcr-abl*, have been targeted with antisense ODNs to inhibit their functions.

c-myb is preferentially expressed in hematopoietic cells. Its down-regulation is associated with increased cell maturation and is required for normal and leukemic hematopoiesis *in vitro* and *in vivo*. The apparent limitation to using antisense ODNs that may affect the proliferation of both normal and leukemic progenitors was circumvented by the demonstration that normal and leukemic hematopoietic cells show differential sensitivity to the effect of *c-myb* antisense ODNs: normal hematopoietic cells survived at doses inhibiting leukemic cell growth.[7] More recently, a series of CML patients in acute and in chronic phase were studied *in vitro* to determine the frequency of elimination of bcr-abl expressing cells following treatment with *c-myb* antisense ODNs. In 8 of the 11 cases treated there was a marked reduction in the colony-forming ability of CML cells. Inhibition was antisense sequence specific, dose dependent and ranged between 58 and 93% of control growth. Furthermore, evaluation of residual colonies for bcr-abl m-RNA expression using reverse transciption-polymerase chain reaction (r-PCR) showed that bcr-abl expression was

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either greatly decreased or undetectable.[8]

The other well-characterized target for antisense inhibition of CML cells is the breakpoint junction on bcr-abl m-RNA transcripts. Two bcr-abl m-RNAs can be transcribed from the fusion gene; in fact, c-abl exon 2 is coupled either with exon 2 (B2A2) or with exon 3 (B3A2) of bcr.[9] Antisense ODNs of different length (16-26 bases), designed against the breakpoint of these two mRNAs, appear capable of inhibiting Philadelphia-positive cells and sparing normal hematopoietic progenitors.

The first demonstration that CML cells might be susceptible to p210 bcr-abl down-regulation was the finding that leukemic cell growth from patients with CML in blastic phase was suppressed by 18-mer antisense ODNs, whereas colony formation from normal progenitors was unaffected. Interestingly, when equal amounts of normal marrow progenitors and blast cells were mixed and exposed to ODNs, the majority of residual colonies were normal.[10] Interest in the potential application of bcr-abl antisense ODNs in clinical settings, at least as *in vitro* purging agents, stimulated other studies aimed at better defining conditions that may influence *in vitro* elimination of leukemic cells by bcr-abl antisense ODNs,[11] or at combining agents with different mechanisms of action to develop more effective purging procedures.[12] The combination, in fact, of low-dose mafosfamide and bcr-abl antisense ODNs caused complete disappearance of bcr-abl transcripts in the experimental conditions used, suggesting very efficient elimination of leukemic cells from bone marrow.

A more clinically relevant use of bcr-abl antisense ODNs would be the treatment of Philadelphia-positive cells from patients in chronic rather than in blastic phase, provided the antileukemic effect of bcr-abl antisense ODNs is still marked on cells from patients in an earlier phase of the disease. In the past few years conflicting results have been generated on the actual elimination of Ph-positive cells from patients with CML in chronic phase.[13-15] Recently, Kirkland *et al.* reported experiments on 15 patients in chronic phase tested with 18-mer and 26-mer antisense phosphorothioate ODNs. Modest non-specific inhibition was observed, highlighted by a similar effect being shown by the corresponding junctional m-RNA antisense and by the alternative junctional antisense ODNs, suggesting that clinical trials of bone marrow purging with bcr-abl antisense ODNs might be premature.[15] In this study, however, incubation of the cells with ODNs lasted only 24-hours, which might be too short to produce a marked specific antileukemic effect. Similarly, the same authors examined the effects of different antisense oligomers on the proliferation of several cell lines. In general, phosphodiester ODNs were inactive, presumably due to degradation by nucleases present in fetal calf serum; however, both B2A2 and B3A2 phosphorothioate antisense ODNs significantly inhibited proliferation of 3 out of 8 cell lines tested. The effect was independent of the type of breakpoint expressed by each cell line, suggesting that the inhibition was sequence dependent but not sequence specific.[16] In this study, the specific down-regulation or degradation of bcr-abl mRNA or protein after exposure to antisense ODNs was not evaluated, and further studies are necessary to delineate the precise mechanisms by which CML cells are inhibited by antisense ODNs.

We analyzed a series of patients with CML in chronic phase using both 16-mer and 26-mer antisense phosphorothioate ODNs (Lynx Therapeutics Inc, Hayward, USA); we evaluated both the duration of incubation and the sensitivity to ODNs of a more immature compartment of CML cells ($CD34^+$ cells) (manuscript in preparation). From our results it seems that 120 hours of incubation and the use of $CD34^+$ concentrated cells are prerequisites for a better antileukemic effect of antisense ODNs, and that patients with CML could be selected for autografting with antisense-purged bone marrow cells on the basis of *in vitro* response.

New aspects in oligodeoxynucleotides research

A number of studies investigated the mechanism of action of ODNs with the aim of increasing antileukemic effect. In a recent work,

the use of fluorescein-labeled phosphorothioate ODNs allowed evaluation of ODN uptake by the BV173 cell line. ODN uptake was dependent on the extracellular concentration and was constant over the first 18 hours of incubation. A decrease in bcr-abl m-RNA was seen only after 3 days of treatment and a large number of apoptotic cells were observed in the antisense-treated group.[17] These data seem to confirm our observation that prolonged incubation is needed to produce effective antisense activity and that perhaps repeated administrations of antisense might be useful in increasing the elimination of rearranged cells.

As a step toward enhancing target selectivity and thereby the differential effects of these reagents on leukemic cells, a DNA delivery system based on receptor-mediated endocytosis was recently adapted to introduce ODNs complexed with a transferrin polylysine conjugate into HL-60 cells.[18,19] Transferrin receptors are abundantly expressed on the surface of tumor cells and, in principle, provide a simple mechanism for the preferential delivery of ODNs in tumor cells. The efficiency of this approach might have been overestimated by the use of a leukemic cell line, in which transferrin receptors are probably much more homogeneously distributed than they are on primary tumor cells; however, the combined use of free and complexed ODNs might be considered since they are likely to utilize different pathways to enter the target cells.

Phosphorothioate ODNs are the most commonly used derivative of unmodified, natural ODNs. They hybridize efficiently to complementary RNA sequences, thereby blocking transcription and translation, and are able to recruit Rnase H, which cleaves the RNA component of the RNA/DNA duplex. On the other hand, phosphorothioate ODNs exhibit sequence-independent mechanisms of activity *in vitro*,[20,21] which may (or may not) have adverse effects *in vivo*. Accordingly, the future development of second-generation classes of modified ODN analogues seems to be likely in the evolution of successful clinical applications in therapies based on the down-regulation of oncogene expression.

Use of antisense oligodeoxynucleotides for ex vivo purging of chronic myelogenous leukemia bone marrow

Autologous bone marrow transplantation is increasingly being used for the treatment of CML, with encouraging results in terms of antileukemic effect and prolongation of survival.[22] Autograft is usually performed with unmanipulated marrow or peripheral blood, since the general view of a *stem cell-involving disease* has hampered the possibility of purging leukemic cells and reinfusing residual normal stem cells. Evidence, however, was recently reported that a normal population of progenitor cells may survive in the majority of Ph-positive CML,[23,24] and several purging strategies have already been developed both experimentally and clinically.[25,26] The demonstration that c-myb or bcr-abl antisense ODNs may inhibit colony formation of CML cells (consequently sparing the normal stem cell counterpart) has stimulated the possible application of antisense ODNs to bone marrow purging.

We started a phase-1 trial on autografting with bcr-abl antisense, ODN-purged marrow in patients with CML in advanced phase.[27] The major aim of this study was to evaluate the antileukemic effect of antisense *in vitro* and the effect of ODN purging on bone marrow engraftment and hematologic reconstitution. For this pilot study we decided not to exceed 24-hour incubation with antisense ODNs in order to avoid the risk of engraftment failure related to *in vitro* manipulations.[28] The concentration of antisense ODNs (150 ug/mL) was chosen on the basis of dose-escalating experiments which demonstrated a lack of toxicity on normal marrow progenitors up to 160 ug/mL of antisense. The results, although preliminary, clearly demonstrated the possibility of purging CML patients *in vitro* with antisense ODNs and engrafting the treated marrow after a time interval similar to that observed in unpurged patients in historical controls.[29]

Autografting with $CD34^+$ marrow cells treated *in vitro* with c-myb antisense ODNs is currently being carried out at the University of Pennsylvania in Philadelphia, USA, under the direction of Dr Alan Gewirtz. Preliminary results in

10 CML patients who underwent this procedure confirm the lack of toxicity of the *ex vivo* treatment, as reflected by the re-establishment of hematopoiesis. In some patients, Philadelphia-positive cells were markedly reduced, raising the possibility of a long-term therapeutic effect of the procedure.[30]

Conclusions

The generation of antisense ODNs specific for gene rearrangements in human cancer has brought renewed optimism to the possibility of specifically treating these genetic abnormality-related malignancies. The ultimate goal of these programs is to develop efficient antisense therapies for the *in vivo* treatment of human leukemias, provided that problems of toxicity, uptake and specificity can be solved. There are, however, several animal studies which may encourage researchers to proceed toward future therapy with antisense ODNs. Recently, the possibility of eradicating leukemia in SCID mice treated systemically with bcr-abl phosphorothioate antisense ODNs was explored.[31] Leukemic mice were given antisense intravenously for 9 consecutive days and evaluated both for the presence of residual leukemic cells in several tissues and for the effect on survival. Bcr-abl transcripts were not detected in six different tissues of antisense-treated mice and all these animals were alive 16 weeks after leukemia cell injection, whereas untreated and sense-treated mice were dead 10-13 weeks after injection.

Before systemic administration of antisense ODNs in patients with CML can begin, appropriate studies of pharmacokinetics, organ distribution and function, some of which have already been conducted in animals, must be performed.[32,33] From the experiments already conducted it would seem that few adverse effects are associated with plasma ODN concentrations that may be therapeutic; furthermore, as far as the cost and production of ODNs are concerned, clinical trials would seem to be feasible. However, the ODN plasma half-life found would indicate that daily administration is preferible, although very little is known about the toxicity associated with chronic ODN infusion. Certainly, before chronic therapy can be considered, issues related to metabolism and reutilization of metabolites must be addressed. As discussed above, the association of ODNs with antiproliferative agents with a different mechanism of action seems to increase the antileukemic activity of bcr-abl ODNs *in vitro*.[12] The possible *in vivo* association of antisense ODNs with agents used regularly for the treatment of CML could be an interesting approach if the appropriate *in vitro* and animal studies are conducted carefully.

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