Ribozyme-Mediated Inhibition of bcr-abl Gene Expression in a Philadelphia Chromosome-Positive Cell Line

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The bcr-abl fusion gene is the molecular counterpart of the Philadelphia chromosome (Ph1) and is directly involved in the pathogenesis of Ph1+ leukemia. Inhibition of bcr-abl expression may have profound effects on the cell biology of Ph1+ cells, as recent experiments with antisense oligonucleotides have shown. In this study we have designed and synthesized a unique ribozyme that is directed against bcr-abl mRNA. The ribozyme cleaved bcr-abl mRNA in a cell-free in vitro system. A DNA-RNA hybrid ribozyme was then incorporated into a liposome vector and transfected into EM-2 cells, a cell line derived from a patient with blast crisis of chronic myelogenous leukemia. The ribozyme decreased levels of detectable bcr-abl mRNA in these cells, inhibited expression of the bcr-abl gene product, p210bcr-abl, and inhibited cell growth. This anti-bcr-abl ribozyme may be a useful tool to study the cell biology of Ph1+ leukemia and may ultimately have therapeutic potential in treating patients with Ph1 leukemias.

Chronic Myelogenous leukemia (CML) arises from the malignant transformation of a pluripotential stem cell and represents about 15% to 20% of all leukemias. The Philadelphia chromosome (Ph1), which results from a reciprocal translocation between chromosomes 9 and 22, is detectable in over 95% of patients with CML. This translocation results in transposition of the cellular abl (c-abl) gene from its usual position on chromosome 9 to chromosome 22.1,2 The breakpoint on chromosome 22 at band q11 occurs within a limited 5.8-kb DNA segment termed the breakpoint cluster region or bcr,3 which resides on a gene composed of 140 kb and 23 exons, now designated the BCR gene.4,5

The transposition of c-abl into the BCR gene results in the creation of an abnormal fusion gene termed bcr-abl, that occurs by fusing either bcr exon 3 to abl exon 2 (splice 1), or bcr exon 2 to abl exon 2 (splice 2). The mRNA transcript that results from the fusion gene is 8.5 kb (±109 bases depending on whether bcr exon 3 is included or not) and is translated into a p210 protein with augmented tyrosine kinase activity. This p210bcr-abl protein and its corresponding mRNA are found in virtually all patients with CML, even those in whom the common t(9;22) translocation is replaced by a variant translocation not apparently involving chromosome 9 or in whom no gross cytogenetic evidence of the Ph1 is present.4,6 The p210bcr-abl is also found in about 50% of patients with Ph1+ acute lymphoblastic leukemia (ALL). In the other 50% of such patients, a p190bcr-abl protein is detected with a correspondingly smaller mRNA of 7 to 7.5 kb. In this fusion gene the first exon of the BCR gene is spliced to the second exon of abl.1,7-9

The bcr-abl gene has been shown to transform hematopoietic cells in vitro, suggesting that it may actually have a causal role in the development of CML.10 As further evidence for the critical role of the p210bcr-abl or p190bcr-abl protein, several investigators have reported that expression of this protein in mice using viral vectors in bone marrow transplant or transgenic mouse models leads to the development of CML- or ALL-like disease in many of these animals.11-15

Ribozymes are a class of RNA molecules that can cleave other RNA sequences enzymatically.16-20 These molecules have two domains: (1) the catalytic “hammerhead” portion that cleaves the target by a mechanism dependent on divalent cations (in particular, magnesium); and (2) the flanking sequences that confer specificity of binding of the ribozyme to the region of the RNA molecule that contains the target sequence. Any sequence of nucleotides of G-U-N, where N = A, C, or U, can be targeted by a ribozyme. In fact, it is possible that any sequence of N-U-N may be a potential target, although some sequences may not be cleaved as efficiently as others. Ribozymes act like enzymes in that one molecule can bind to its RNA target, cleave it, then dissociate and bind to a second target, and so on.

Ribozymes have tremendous potential to specifically inhibit the expression of a variety of genes and represent a novel therapeutic approach to controlling viral infections and oncogenesis. Researchers have begun to apply this technology to inhibit expression of human immunodeficiency virus genes,21,22 as well as c-fos23 and H-ras oncogenes in vitro and in animal models.

This approach is similar in some ways to the use of antisense molecules to inhibit expression of viral genomes, growth factors, and oncogenes.24-32 For example, Anfossi et al33 and Kamano et al34 have used c-myb antisense oligodeoxynucleotides (oligos) to inhibit the growth of human myeloid leukemia cell lines and to demonstrate differential sensitivity to such inhibitory effects between normal and leukemic hematopoietic cells.35,36 These investigators have also used antisense oligos directed against the bcr-abl oncogene to inhibit cell growth of freshly isolated leukemic blasts from CML patients.37

The aim of this study was to use ribozyme technology to
inhibit expression of this bcr-abl gene in Ph1+ leukemia cells and to evaluate the effect of this inhibition on cell proliferation. Specific ribozymes were synthesized both as RNA and as hybrid DNA-RNA oligonucleotides (oligos). The catalytic activity of the ribozyme was then analyzed both in a cell-free system and after transfection into a Ph'+ cell line, EM-2, using liposome vectors. The effects of the anti-bcr-abl ribozyme on bcr-abl gene expression and on EM-2 cell proliferation were then evaluated.

MATERIALS AND METHODS

Synthesis of anti-bcr-abl ribozyme. A “hammerhead” ribozyme to cleave bcr-abl mRNA splice 1 (Fig 1) was synthesized in the City of Hope DNA Synthesis Core facility. Ribozyme cDNA was subcloned to a pBluescript vector (Stratagene, La Jolla, CA) which was used to generate RNA ribozyme using T7 RNA polymerase in vitro. To increase the stability of the ribozyme to ribonucleases, the ribonucleotides (RNA) in the flanking arms and in a portion of the loop structure were replaced with deoxyribonucleotides (DNA). This chemically synthetic construct is referred to as DNA-RNA hybrid anti-bcr-abl ribozyme (DH-Ribo). As controls for the ribozyme, a ribozyme directed against totally unrelated sequences of mRNA (Unrelated Ribo), bcr-abl sense, and bcr-abl antisense oligos were tested. The sequences of these oligos in 5' to 3' orientation are as follows (bases in bold letters represent ribonucleotides; all other bases are deoxyribonucleotides): anti-bcr-abl ribozyme: CUGAAGGGCUUUUGAGAGGUGCGUGAGGAGGGAC-CAACUGCUUAAAGCCCGCG; DH-Ribo: GGCTTTGCUAGGACGAAATCGCTCTTTG; bcr-abl sense: AGAGATTCAAAGGCGTCTTTTGAGCCAAGAACCTT; bcr-abl antisense: GAAGGGCTTTGAGCCCGCGCTTCCAGAGAGT; Unrelated Ribo: TACCTTGUGAUAGGTCTGCGTGAGGACGAAAAGTCCAGTCG.

Preparation of target bcr-abl splice 1 mRNA substrate. The pJWP3 plasmid containing bcr-abl splice 1 cDNA was kindly provided by Dr Owen Witte (University of California at Los Angeles). This cDNA was incorporated into the pBLUESCRIPT-KS(+) vector (Stratagene, La Jolla, CA), subcloned, and transcribed to RNA using T7 RNA polymerase and [32P]-UTP as described by Lowary et al.60

Cell-free assay of ribozyme cleavage of bcr-abl mRNA. The in vitro ribozyme cleavage reactions were performed as described previously.24 In brief, the in vitro ribozyme cleavage reactions were performed in 20-μL volume containing 2 pmol P2-labeled bcr-abl target RNA in 75 mmol/L Tris-HCl pH 7.5 and 1 mmol/L NaEDTA. The mixture was heated to 72°C for 2 minutes followed by rapid cooling on ice. To initiate the reaction, MgCl2 was added to 10 mmol/L, and the mixture was incubated for either 3 hours or 12 hours at 37°C. The reaction was stopped by the addition of formamide-EDTA dye, and the resulting mixture was analysed by 7 mol/L urea 8% polyacrylamide gel electrophoresis (PAGE).

Ribozyme transfection of EM-2 cells. The EM-2 cell line (gift from Dr Owen Witte) is maintained in RPMI with 10% fetal calf serum (FCS) in our laboratory. During transfection, the EM-2 cells were washed two times with Opti-MEM reduced serum medium. Three additional doses of either oligos or ribozymes with 20 μg/mL Transfectam (Lipopolyamine; Promega, Madison, WI) were added to the wells and incubated for 4 hours at 37°C. At 4 hours additional oligos or ribozymes were added without Transfectam to increase their final concentration to 4 μmol/L. FCS, 65°C inactivated, was added to each well to yield a final concentration of 4% in Opti-MEM reduced serum medium. Three additional doses of 2 μmol/L of oligos or ribozyme were then added at 24, 36, and 72 hours. Uptake of [32P]-labeled ribozyme with Transfectam at 2 hours was 26%, compared to 9% without it.
mRNA of bcr-ab1

**Fig 3.** Autoradiogram of bcr-ab1 mRNA extracted from EM-2 cells and analyzed by ribonuclease protection assay after transfection in Transfectam liposomes with the various oligos indicated. See Results for quantitative results from densitometric scanning. Control EM-2 cells in lane 1; EM-2 cells transfected with: bcr-ab1 sense oligo in lane 2, bcr-ab1 antisense oligo in lane 3, DH-Ribo in lane 4.

**Cell proliferation assays.** EM-2 cell counts and viability (trypan blue exclusion) were determined daily until the fourth day. Four hours after the third addition of oligos or ribozyme to the cultures (ie, at 76 hours), EM-2 cells (2 x 10^6) were directly seeded into duplicate methylcellulose dishes in the absence of hematopoietic growth factors and incubated for 10 days at 37°C, 5% CO2, with 15% FCS. The plates were then scanned for total numbers of colonies.

**RNA analysis.** EM-2 cells exposed for 48 hours to oligos or ribozymes were extracted for total cellular RNA by using guanidium isothiocyanide denaturing solution (RNAzol; Cinna-Biotex, Houston, TX). Bcr-ab1 mRNA was analyzed by ribonuclease protection assay RPA II (Ambion, Austin, TX), then scanned densitometrically by AMBlS systems (San Diego, CA). The 32P-labeled bcr-ab1 RNA probe used in this assay was synthesized from the opposite orientation of pBLUESCRIPTbcr-ab1 vector by T3 RNA polymerase.

**Immunoprecipitation and protein kinase assay of p21bcr-ab1 protein.** EM-2 cells treated with either oligos or ribozymes for 72 hours were extracted for cellular proteins. The assays were performed as described.31 Cell lysates were immunoprecipitated with anti-bcr monoclonal antibody (bcr-Ab2, Oncogene Science, Manhasset, NY). Immune complexes were collected on protein A sephrose beads (Pharmacia Fine Chemicals, Piscataway, NJ) and kinase reactions performed in the presence of 32P-labeled adenosine triphosphate for 20 minutes at 37°C. The sizes of the autophosphorylated bcr and bcr-ab1 proteins were determined by 7% sodium dodecyl sulfate-PAGE (SDS-PAGE) followed by autoradiography.

**Statistical methods.** Student's t-test was used to determine the significance of differences in mean cell numbers in the cell growth experiments.

**RESULTS**

**Cell-free cleavage of bcr-ab1 mRNA.** As shown in Fig 2, the anti-bcr-ab1 ribozyme cleaved bcr-ab1 mRNA splice 1 in an Mg++-dependent reaction. There was a 69% decrease in the amount of substrate remaining at 12 hours compared with a 25% decrease after 3 hours of incubation. The two cleavage products labeled as P1 and P2 are the correct sizes predicted from the location of the cleavage site for the ribozyme, as shown in Fig 1. There is some degradation of the ribozyme after 12 hours in the presence of Mg++.

**Resistance of ribozyme to RNase.** Both the RNA and DNA-RNA hybrid ribozymes were incubated for 1 hour at 37°C with RNase at varying concentrations to assess the relative stability of the hybrid ribozyme. The RNA ribozyme was completely destroyed by RNase at 1 µg/mL. In contrast, approximately 50% and 10% of the hybrid ribozyme remained intact at RNase concentrations of 1 µg/mL and 5 µg/mL, respectively (data not shown).

**Ribozyme effect on bcr-ab1 mRNA expression.** Figure 3 shows the results of a ribonuclease protection assay using a 32P-labeled bcr-ab1 RNA probe to detect bcr-ab1 mRNA extracted from EM-2 cells that had been incubated with the hybrid ribozyme or with various control oligos. Quantitative densitometric scanning of the autoradiogram showed a 49% decrease in detectable bcr-ab1 mRNA from EM-2 cells,

**BCR PROTEINS**

![Autoradiogram of bcr proteins extracted from HeLa cells and EM-2 cells, analyzed by immunoprecipitation/protein kinase assay. The major band in all lanes is the p160 bcr protein. Smaller bcr proteins are also seen. The p210bcr-ab1 protein is expressed only in EM-2 cells, and its expression is completely inhibited by the DH-Ribo. Lane 1, HeLa cells; lane 2, control EM-2 cells. EM-2 cells transfected with: bcr-ab1 sense oligo in lane 3, bcr-ab1 antisense oligo in lane 4, DH-Ribo in lane 5.](image)
incubated with the DH-Ribo compared with control cells and a 25% decrease in cells incubated with antisense oligo. Expression of β-actin genes was not inhibited by the ribozyme or other oligos (results not shown).

Ribozyme effect on p210(bcr-abl) protein synthesis. Figure 4 shows the results of an immunoprecipitation/protein kinase assay to detect the family of bcr proteins extracted from control HeLa cells and from EM-2 cells incubated with the hybrid ribozyme or the various control oligos. The major band in all groups is the main p160 bcr protein. Multiple bands of smaller molecular weight bcr proteins are also detected (eg, p135, p108, p83). Only the EM-2 cells express the p210(bcr-abl) protein. Expression of this protein was completely inhibited by the DH-Ribo. A faint p210 band is visible after incubation with the antisense oligo. The intensity of the p160 band in each lane serves as an internal control for the total amount of protein loaded.

Ribozyme effect on EM-2 cell growth. The effect of anti-bcr-abl ribozyme on EM-2 cell growth was assessed both in liquid cultures and in methylcellulose colony formation assays. Figure 5 shows that in three similar experiments performed, the DH-Ribo inhibited EM-2 cell growth by 84%; the antisense oligo inhibited growth by 71%. These inhibitory effects were statistically significant by t-test both when antisense and DH-Ribo were compared with control and when antisense was compared with DH-Ribo. There was no significant inhibition by the liposome vector alone, sense oligo, or unrelated ribozyme.

Percent inhibition of cell growth was calculated as follows: \[ \% \text{inhibition} = \left( \frac{\text{Exp}_{\text{DH-Ribo}} - \text{Exp}_{\text{Control}}}{\text{Exp}_{\text{Control}}} \right) \times 100; \]

where \( \text{Exp}_{\text{DH-Ribo}} \) is cell count for experimental group at Day 4; \( \text{Exp}_{\text{Control}} \) is cell count for experimental group at Day 1; \( \text{Exp}_{\text{Control}} \) is cell count for control group at Day 1.

Table 1 shows the results of a representative experiment of EM-2 colony formation in methylcellulose cultures. DH-Ribo inhibited colony formation dramatically, whereas the Transfectam liposome alone and an unrelated ribozyme had only minimal effects.

**DISCUSSION**

The p210(bcr-abl) protein and its corresponding mRNA are found in virtually all patients with CML. Results from in vivo10 and mouse model studies12,13 strongly support the pathogenic role of this oncogene in the development of Ph+ leukemia.

The close proximity of the GUU nucleotide sequence to the bcr-abl mRNA fusion site suggested that a specifically designed ribozyme could be used to cleave the abnormal mRNA sequence. We have shown in this study that a ribozyme designed against the bcr-abl mRNA splice 1 can cleave this substrate in a cell-free system. More importantly, after transfection into a Ph+ cell line, the ribozyme inhibits expression of both bcr-abl mRNA and the p210(bcr-abl) protein product and can inhibit growth of these cells in suspension and in methylcellulose cultures.

Ribozymes have been shown to have tremendous po-
tial to inhibit the expression of a variety of genes and, thus, may represent a novel therapeutic approach to controlling viral infections and oncogenesis. This approach is similar to the use of antisense oligos to inhibit expression of viral genomes, growth factors, and oncogenes. For example, Szczylak et al. have used antisense oligos directed against the bcr-ab1 oncogene to inhibit cell growth of freshly isolated leukemic blasts from CML patients. Ribozymes appear to act both as antisense oligos and as catalytic enzymes that cleave their substrate. A single ribozyme molecule can inactivate its target mRNA sequence by cleaving it, then dissociate from the two products, bind to a new target molecule, inactivate it, and so on. It should be noted that antisense RNA probes may also rehybridize with new substrate molecules and, thus, recycle. It was not our intention in this study to compare the inhibitory activity of anti–bcr-ab1 ribozymes directly with that of antisense oligos. It is difficult to control the intracellular concentrations of ribozyme and antisense molecules using our transfection method. Such a comparison may be more valid in a transduction model using a viral vector that allows for stable, long-term expression of these molecules.

We have used a liposome vector to transfect target cells with a ribozyme directed against bcr-ab1 mRNA. This approach is relatively inefficient and the expression of the ribozyme is short-lived. Recently, we have successfully cloned the anti–bcr-ab1 ribozyme gene into an adeno-associated viral (AAV) vector to achieve long-term, constitutive expression of the gene encoding the ribozyme in transfected cells. In such a system we will be able to more accurately assess the efficacy of the ribozyme in blocking bcr-ab1 gene expression in cell lines and in freshly isolated leukemic cells. We will also be able to compare directly the relative efficiencies of anti–bcr-ab1 ribozyme and antisense molecules by transducing target cells with AAV constructs that contain and stably express either the ribozyme or antisense gene.

Our data showing that a ribozyme designed specifically against bcr-ab1 mRNA can inhibit expression of this gene and cell growth of a Ph+ cell line further support the pathogenic role of the bcr-ab1 gene in Ph+ leukemia. In addition, anti–bcr-ab1 ribozymes may have therapeutic potential in the treatment of patients with Ph+ leukemia using genetically modified marrow, leading to specific inhibition of leukemic cell proliferation without affecting normal hematopoietic precursors.

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