Multi-Unit Ribozyme-Mediated Cleavage of bcr-abl mRNA in Myeloid Leukemias

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Chronic myelogenous leukemia is characterized by the Philadelphia chromosome, which at the molecular level results from the fusion of the bcr gene on chromosome 22 and the abl gene on chromosome 9. The bcr-abl fusion gene encodes a novel tyrosine kinase with transforming activity. In this study, we have synthesized a multi-unit ribozyme that targets bcr-abl mRNA. In vitro ribozyme cleavage reactions show increased cleavage efficiency of this multi-unit ribozyme compared with single or double ribozymes. The multi-unit ribozyme was then transfected into murine myeloblasts transformed with the bcr-abl gene (32D cells). Ribozyme transfection was accomplished either by liposomes or using folic acid-polylysine as a carrier. Multi-unit ribozyme transfection reduced the level of bcr-abl mRNA 3 logs when transfected via folate receptor-mediated uptake into transformed 32D cells. These results suggest that a multi-unit ribozyme could be an effective therapeutic agent for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia.

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Supported by the American Society of Clinical Oncology Young Investigator Award and Career Development Award (L.H.L.), the W.W. Smith Charitable Trust (S.K.S.), and National Institutes of Health Grant No. 1 ROI, 5POICA21124-15 (E.P.R.).

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0006-4971/95/8508-0002$3.00/0

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The conjugation number was determined by measuring the optical density at 363 nm of the conjugate solution, and the conjugation number was transfection mix was added to 4 cells.

The protocols described by Reddy et al were used. The folate conjugates in 50 μL of water and gently mixed for 15 minutes. The transfection mix was added to 4 cells.

Liposomes containing ribozyme RNA were prepared in 100 volumes. All RNA was suspended in 50 mmol/L Tris-HCl, pH 7.5, containing 1 mmol/L EDTA and was heated to 95°C for 5 minutes and immediately chilled on ice. Reactions were initiated by adding 1 μL of 200 mmol/L MgCl2 and stopped after 2 hours by adding 2 μL of stop solution containing 95% formamide, 20 mmol/L EDTA, and 2% bromophenol blue. Cleavage products were analyzed by electrophoresis on a 6% denaturing gel followed by autoradiography and quantitation densitometry (Fujix, Tokyo, Japan). The kinetics of ribozyme cleavage were identical in all experiments by removing 1 μL of the reaction mixture at specified times and adding 1 μL of stop solution. Cleavage products were analyzed as above.

32D cell transformation. 32D cells, a murine BM myeloblast cell line, was maintained in Iscove's modified Dulbecco's media (IMDM) with 10% fetal bovine serum (FBS), and 10% WEHI-322 supernatant as a source of murine interleukin-3 (IL-3). Retroviral expression vectors expressing the bcr-abl gene along with neomycin resistance gene were introduced into 32D cells by electroporation. A total of 10 μg of linearized DNA was electroporated into 32D cells using 300 V and 960 microfarads using the BioRad Gene Pulser (BioRad, Hercules, CA) according to the manufacturer's protocol. Transformed cells were selected by growth in L-3-deficient medium, containing 1 μg/mL of G418, and immediately chilled on ice. Reactions were initiated by adding 1 μL of 200 mmol/L MgCl2 and stopped after 2 hours by adding 2 μL of stop solution containing 95% formamide, 20 mmol/L EDTA, and 2% bromophenol blue. Cleavage products were analyzed by electrophoresis on a 6% denaturing gel followed by autoradiography and quantitation densitometry (Fujix, Tokyo, Japan). The kinetics of ribozyme cleavage were identical in all experiments by removing 1 μL of the reaction mixture at specified times and adding 1 μL of stop solution. Cleavage products were analyzed as above.

Ribozyme design, synthesis, and packaging. To develop ribozyme-based reactions as a potential therapy for human
leukemias, we have designed hammerhead motifs that can cleave specifically bcr-abl mRNA, thereby preventing the synthesis of the transforming fusion protein. Single-, double-, and triple-unit ribozymes were designed and constructed targeting GUX sequences near the bcr-abl fusion site (Fig 1). Single-unit ribozymes were synthesized as cDNA oligomers, whereas double- and triple-unit ribozyme expression vectors were produced by ligation of single-unit ribozyme cDNAs. Nonfunctional ribozymes (antisense controls), with a G substituted for the A in the conserved GAG sequence (marked with an asterisk in Fig 1) in the catalytic unit eliminating catalytic function, were similarly cloned and transcribed. Sense ribozymes were synthesized by transcription from the opposite strand in pGEM3z vector using T7 RNA polymerase.

In vitro ribozyme cleavage reactions. To test the efficiency of single-, double-, and triple-unit ribozymes, we performed in vitro cleavage reactions. Four single-unit ribozymes, two double-unit ribozymes, and one triple-unit ribozyme, whose structures are shown in Fig 1, were used in these studies. A constant amount of 32P-labeled substrate bcr-abl RNA was incubated with equal molar amounts of various ribozymes for 2 hours, and the cleavage products were analyzed on a denaturing polyacrylamide gel as described in Materials and Methods. As shown in Fig 2A, single-, double-, and triple-unit ribozymes specifically cleave bcr-abl mRNA. After 2 hours, single ribozymes cleaved target bcr-abl mRNA into 2 fragments with variable efficiency. Ribozymes A, B, C, and D cleaved 45%, 79%, 5%, and 7% of target mRNA, respectively. Comparison among these ribozymes shows that ribozymes targeting the same site but with shorter annealing arms are more efficient (A v C) and GUC cleavage sites (ribozyme B) appear to be more efficiently cleaved. The variability in cleavage of substrate RNA by ribozymes B, C, and D is not surprising and reflects the expected cleavage efficiency at different GUX cleavage sites. Double-unit ribozymes E and F cleaved 72% and 13% of target mRNA, respectively. There was no significant improvement in cleavage over the best single ribozyme of the pair. However, ribozyme F, the triple-unit ribozyme, cleaved 95% of target mRNA in 2 hours. Figure 2B shows the cleavage obtained when all 3 single unit ribozymes are added together in one reaction. This resulted in little additional cleavage compared with that of the best single ribo-

![Fig 2. Autoradiogram of ribozyme-mediated cleavage of bcr-abl mRNA.](from www.bloodjournal.org)
RIBOZYME-MEDIATED THERAPY OF CML

Fig 3. Comparison of triple-unit and single-unit ribozyme cleavage kinetics. Equal molar concentrations of \(^{32}\)P-labeled substrate bcr-abl mRNA and ribozyme G or C were mixed at time zero. Aliquots of the reaction mixture were removed and placed in stop solution at the specified time points. Cleavage products were separated on a 6% denaturing gel and percentage of cleavage was determined by quantitative densitometry.

zyme (ribozyme B). There was no cleavage of bcr-abl mRNA using sense-oriented ribozymes and cleavage-inactive antisense ribozymes (data not shown).

The kinetics of in vitro cleavage are shown in Fig 3. As described in Materials and Methods, equal molar amounts of \(^{32}\)P-labeled bcr-abl substrate was incubated with single- or triple-unit ribozymes for various times, and aliquots were analyzed over time for percentage of cleavage. Ribozyme C was used as the single-unit ribozyme for comparison because its sequence was contained within the triple-unit ribozyme and targets the bcr-abl junction. Ribozymes B and D target normal bcr and abl sequences and would not be specific for bcr-abl sequences. These ribozymes would have little clinical utility. By 30 minutes, cleavage of target mRNA by the triple-unit ribozyme is nearly complete, whereas the single ribozyme has had little effect on the same target mRNA.

To test the specificity of the triple-unit ribozyme, c-abl and c-bcr mRNA containing the breakpoint region was transcribed with \(^{32}\)P-CTP and used as a target for single- and triple-unit ribozymes in similar 2-hour cleavage reactions. Figure 4 shows specific cleavage products from these reactions. In Fig 4A, ribozyme D cleaved 3% and ribozyme G cleaved 87% of c-abl mRNA. In Fig 4B, ribozyme B cleaved 83% and ribozyme G cleaved 45% of target bcr mRNA. Thus, the more efficient triple-unit bcr-abl ribozyme does cleave c-abl and bcr mRNA, although less efficiently as compared with bcr-abl sequences.

Ribozyme transfection of transformed 32D cells. Having shown that the triple-unit ribozyme efficiently cleaved bcr-abl mRNA, we next compared two transfection techniques to standardize a transfection protocol for use in subsequent experiments. For comparison, we used either lipofectin2" or folic acid-polylysine28 because these two reagents have been reported to yield high levels of transfection. The transfection protocols were standardized by varying both the concentration of transfection vehicle and RNA over 4 logs and the incubation time. After 3 hours of ribozyme exposure, total cellular RNA was isolated from bcr-abl-transformed 32D cells and was analyzed by electrophoresis on a 6% sequencing gel followed by autoradiography and quantitative densitometry. Figure 5A shows the improvement in \(^{32}\)P-labeled ribozyme uptake in 32D cells that results by incorporating the ribozyme in a liposome. Naked multi-unit ribozymes are poorly taken up by cells (Fig 5A, lane 1), whereas liposomes augmented delivery 100-fold (Fig 5A, lane 2). In addition to isolating total cellular RNA and comparing ribozyme delivery, the supernatant from these transfection experiments was monitored for ribozyme stability. Naked ribozymes were degraded over several hours, whereas ribozymes packaged in liposomes remained stable in serum-free medium for 24 hours (data not shown). Analysis of cellular RNA after 3 hours of ribozyme exposure insured that uptake comparisons were made during the period of time in which ribozymes were present under all transfection conditions.

Figure 5B compares liposome-mediated and folate receptor-mediated transfection techniques in 32D cells. Using 1 \(\mu\)g of \(^{32}\)P-labeled ribozyme, cells were transfected with different concentrations of lipofectin or folic acid-polylysine.
that varied over 4 logs. Ribozyme uptake was more efficient (sevenfold) by folate receptor-mediated transfection. Similar to liposome vectors, folic acid-polylysine vectors protected ribozymes from being degraded in serum-free medium (data not shown).

**Transfection standardization.** To further optimize the transfection conditions, a series of experiments were performed to determine the dose-response relationship between ribozyme and transfection vehicle concentration in 32D cells. First, 1 x 10⁶ cells were transfected with 1 µg of ³²P-labeled triple-unit ribozyme via increasing concentrations of liposomes or folic acid-polylysine. Next, based on these dose-response curves, the quantity of transfection vehicle was held constant and the quantity of ribozyme was varied. Then, using optimum ratios of ribozyme and transfection vehicle, the time course for ribozyme uptake and survival was determined. Figure 6 graphically shows the results from these experiments in 32D cells. Although both transfection techniques augmented ribozyme uptake, folate receptor-mediated transfection improved uptake up to 10-fold. Furthermore, multi-unit ribozyme RNA remained at significant intracellular levels in 32D cells after 24 hours. This occurred despite the addition of FBS to the culture medium after 3 hours, which quickly degraded ribozyme RNA in the medium (data not shown). In subsequent transfection experiments either, 5 µg of lipofectin was diluted in 50 µL of water and mixed with 1 µg of RNA suspended in 50 µL of water or 1 µL of folic acid-polylysine was diluted in 50 µL HEPES-buffered saline and mixed with 1 µg of RNA suspended in 50 µL HEPES-buffered saline was used.

**Serial transfections.** To test the availability of the folate receptor for serial transfections, 32D cells were serially transfected with unlabeled and ³²P-labeled ribozymes over a 48-hour period. Three identical folic acid-polylysine transfection experiments were performed at time zero with either ³²P-labeled (one sample) or unlabeled ribozymes (two samples). After 3 hours, the cells transfected with labeled ribozymes were lysed and RNA was isolated, whereas cells transfected with unlabeled ribozymes were supplemented with FBS and IL-3 as described above. After 24 hours, one of the two remaining samples was then washed in purified buffered saline and transfected with ³²P-labeled ribozyme. After 3 additional hours, these cells were washed and lysed, and RNA was isolated. A second transfection with unlabeled ribozymes was performed on the remaining cells (second dose), and these cells were supplemented as described. After 48 hours, these cells were transfected with labeled ribozymes (third dose), and, after 3 hours, RNA was isolated as described above. The RNA samples were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel electrophoresis followed by autoradiography and quantitative densitometry. Figure 7 shows the autoradiogram that demonstrates that serial folic acid-polylysine transfection results in a decrease in ribozyme uptake. Compared with the quantity of ³²P-labeled ribozyme recovered from cells at 3 hours, 50% was recovered after the second transfection, and 60% was recovered after the third transfection. These experiments show that less ribozyme is delivered during sequential folate receptor-targeted transfections in 32D cells, probably because of receptor occupancy or downregulation of receptor density on the cell surface.

**RNA analysis.** To test the ability of the multi-unit ribozyme to cleave bcr-abl mRNA in cells, untransformed 32D cells were added to various numbers of bcr-abl--transformed 32D cells and were transfected 1 or 3 times over 24 or 48 hours respectively, with triple-unit ribozyme via liposomes or folic acid-polylysine vectors. Total cellular RNA was isolated after 24 and 48 hours and was reverse-transcribed into cDNA. The cDNA was amplified by PCR using primers that amplify the bcr-abl breakpoint. Purified cellular RNA was DNA-free, and there was no amplification of DNA in the absence of RT, suggesting only reverse-transcribed RNA is being amplified (data not shown). Figure 8 shows the results of a Southern blot performed on the PCR products using an oligonucleotide probe that spans the bcr-abl breakpoint. Control cells transfected with folic acid-polylysine vectors alone show that this technique has a sensitivity for detecting 1 transformed cell in 1 x 10⁶ normal cells. A single transfection of the triple-unit ribozyme via folic acid-polylysine vectors into bcr-abl--transformed 32D cells resulted in a 3-log
decrease in detectable \( \text{bcrl-ABL} \) mRNA signal 24 hours after transfection. There was no improvement in reducing the \( \text{bcrl-ABL} \) mRNA with multiple transfections over 48 hours compared with single transfections (data not shown). A 1-log reduction in \( \text{bcrl-ABL} \) RNA content was observed by transfection of ribozymes via liposomes, despite multiple treatments over 48 hours (24 hour data shown in Fig 8). Cleavage-inactive ribozymes had no effect on elimination of the \( \text{bcrl-ABL} \) mRNA signal (data not shown). RT-PCR using primers that amplified the \( \beta \)-actin gene confirmed that similar quantities of RNA were used in RT-PCR amplification for the \( \text{bcrl-ABL} \) mRNA signal (data not shown). These results show that delivery of a triple-unit ribozyme using folic acid-polylysine reduces the levels of \( \text{bcrl-ABL} \) RNA by 3 logs, whereas only a 1-log reduction is achieved with liposomes. These results are consistent with the levels of ribozyme present in cells after transfection using these two reagents.

**DISCUSSION**

CML is characterized by the presence of Ph chromosome that, at the molecular level, results from the fusion of the \( \text{BCR} \) gene on chromosome 22 with the \( \text{ABL} \) gene on chromosome 9.\(^6\) This results in the generation of an 8.5-kb chimeric mRNA and an abnormal p210\(^{\text{BCR-ABL}}\) tyrosine kinase.\(^4\) This appears to be the transforming signal that leads to the development of CML.\(^9\)

There are no conventional therapies that have resulted in cures in CML, including chemotherapy, \(^{30}\)P,

and splenic irradiation.\(^{31}\) In 5% to 15% of patients, \( \alpha \)-interferon therapy may suppress the expression of the Ph-positive clone in CML.\(^{32,33}\) However, the ability of interferon to cure CML has not been shown. Allogeneic BMT, using HLA-identical siblings, after myeloablative chemoradiotherapy is curative in up to 85% of carefully selected patients in chronic phase CML.\(^{34-36}\) In CML patients transplanted with BM grafts from an identical twin after high dose myeloablative therapy, the cure rate is 50%. This difference is because of a graft-versus-leukemia (GVL) effect and is enhanced by the presence of chronic graft-versus-host disease. T-cell depletion of donor BM reduces both the GVL effect and the incidence of graft-versus-host disease, as well as the cure rate.\(^{34,35}\) Collectively, these data show that high-dose therapy administered early in the course of CML is potentially curative in 50% of patients. The additional curative effects of allogeneic BMT are because of a GVL effect.

Less than 30% of CML patients will have a normal allogene-
Ribozyme -

Fig 7. Autoradiogram from sequential transfections of ribozymes via folate receptor-mediated uptake in 32D cells. To test the availability of the folate receptor for serial transfections, 32D cells were serially transfected with unlabeled and 32P-labeled ribozymes over 48 hours. Total cellular RNA was isolated 3 hours after each transfection experiments. Lane 1 contains RNA from cells transfected at time zero with 32P-labeled ribozyme. Lane 2 contains RNA from cells transfected at time zero with unlabeled ribozyme followed by 32P-labeled ribozyme at 24 hours. Lane 3 contains RNA from cells transfected at time zero and 24 hours with unlabeled ribozyme followed by 32P-labeled ribozyme at 48 hours.

Similar results have been described for a nonaribozyme targeting HIV-1 env RNA. An important consequence of targeting normal sequences of the bcr and abl genes in the proximity of the fusion site is that normal mRNA may be affected by the ribozyme. Although we observe cleavage of normal bcr and abl mRNA, this appears to be less efficient in vitro cell-free cleavage experiments than bcr-abl cleavage. This could be caused by extensive nonhomology of portions of the ribozyme to normal abl and bcr RNAs that, in turn, could reduce the affinity of the ribozyme to the substrate.

To develop a protocol for ribozyme-mediated treatment of human CML, both efficiency of delivery and ease of application need to be taken into consideration. To accomplish rapid and efficient delivery of ribozymes into myeloid cells, we used three different methods. The first method tried was the receptor-mediated uptake of naked oligonucleotides, which proved to be extremely inefficient, possibly because of the delivery of the nucleotide molecules via the endosomal pathway, which exposes the ribozyme molecules to various nucleases of the cell. We next studied nontargeted uptake via cationic lipids and receptor-mediated uptake via the folate receptor. Both augmented ribozyme delivery to 32D cells, although folate-receptor-mediated uptake was more efficient. However, only folate acid-polylysine-mediated delivery of the ribozymes seemed to affect bcr-abl mRNA levels significantly. Although we cannot rule out a quantitative effect because of improved delivery of ribozymes via folate receptor uptake, the intracellular compartment accessible to ribozymes may be more favorable by this technique. As reviewed by Gottschalk et al., folate enters cells through several different receptor systems, including a glycosyl-phosphatidyl-anchored protein that is excluded from clathrin-coated pits and cycles in and out of cells by caveolae. RNA transacted via liposomes may be quickly degraded, because this uptake is mediated via an endosomal

Cell number

+ - 1 10 10^2 10^3 10^4

Control -

Folate transfections -

Liposome transfections -

Fig 8. Autoradiogram of a Southern blot of RT-PCR-amplified bcr-abl mRNA from transformed 32D cells transfected with ribozymes via liposomes or folate acid-polylysine vectors. From 1 to 1 x 10^6 bcr-abl-transformed 32D cells were added to 1 x 10^6 untransformed 32D cells. Cells were transfected with ribozymes or vectors containing no ribozymes (controls), and, after 24 hours, total cellular RNA was extracted. RT-PCR was performed with primers that amplified the bcr-abl chimeric gene and the β-actin gene (an internal control). Southern blotting was performed with a 32P-kinased probe that detects the bcr-abl breakpoint. + control, RNA transacted from a plasmid containing the bcr-abl breakpoint region (see Materials Methods); - control, no template addition.
pathway, which exposes the ribozymes to cellular nucleases. On the other hand, receptor-mediated uptake may protect the ribozymes from intracellular degradation caused by exposure to cellular nucleases. Folate-mediated uptake may suffer from a first-dose effect if receptors are downregulated after ligand binding. In such an event, the problem could be circumvented by targeting different receptors sequentially. Our results show that, although transfection efficiency decreases with serial transfections, ribozymes continue to be delivered into cells when transfections are separated over a 24-hour time period.

The ultimate measure of ribozyme effects on bcr-abl mRNA are decreasing the bcr-abl mRNA signal, reducing p210 bcr-abl protein levels, and reversing the transformed phenotype in living cells. Our results show a reduction in bcr-abl mRNA levels in transformed 32D cells when transfected with ribozymes delivered by folate receptor-mediated uptake. Unfortunately, multiple sequential transfections did not improve on the results obtained by a single transfection over 24 hours. It is possible that constitutive expression of ribozymes transfected by retroviruses in a similar model may show greater efficacy.

ACKNOWLEDGMENT

This paper is dedicated to the memory of T.A. Newkirk. He was a dedicated scientist and good friend.

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Multi-unit ribozyme-mediated cleavage of bcr-abl mRNA in myeloid leukemias

LH Leopold, SK Shore, TA Newkirk, RM Reddy and EP Reddy