Reversal of Drug Sensitivity in Multidrug-Resistant Tumor Cells by an MDR1 (PGY1) Ribozyme

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ABSTRACT

In order to reverse P-glycoprotein-mediated drug resistance in a specific manner, we designed two hammerhead ribozymes which can cleave the GUC sequence in codon 179 and 196 of MDR1 (PGY1) mRNA. The ribozymes were directly synthesized using a set of primers, one containing a bacteriophage T7 RNA polymerase promoter. A target MDR1 RNA was created by a reverse transcription polymerase chain reaction using a MOLT-3 human acute leukemia cell line resistant to trimetrexate (TMQ) (MOLT-3/TMQ800), which displayed MDR1 overexpression. In a cell-free system, both ribozymes cleaved a target piece of MDR1 RNA into 2 fragments at the specific sites at a physiological pH and temperature. The cleavage reaction was dependent on time, ribozyme:substrate ratio, and magnesium concentration. The 196 MDR1 ribozyme was more active than the 179 MDR1 ribozyme. The 196 MDR1 ribozyme was then cloned into a T7 RNA polymerase promoter. RNAs of size 329 bases were obtained and used for cleavage reaction in a cell-free system (19).

INTRODUCTION

Tumor cells exposed to natural hydrophobic cytotoxic drugs may become cross-resistant to a series of structurally and functionally unrelated drugs. This phenomenon, called MDR, is one of the major obstacles in cancer chemotherapy. One of the underlying mechanisms of MDR is the cellular overproduction of P-glycoprotein, a 170-kDa transmembrane protein that can act as a drug efflux pump (1-3). P-glycoprotein is encoded by a small group of closely related genes termed MDR (4-8). Theoretically, P-glycoprotein activity can be blocked at three levels: DNA, RNA, or protein. Since amplification of MDR1 gene is not a prerequisite to P-glycoprotein-related resistance in human tumor cells (9-11), the potential loci of inhibition can be narrowed to mRNA. One means to intercept a target mRNA is to use a catalytic RNA, or ribozyme (12, 13). Since ribozymes recognize a GUC sequence and cleave it most efficiently, attempts were made to determine whether a GUC sequence is located within any critical region on MDR1 mRNA. There are 37 GUC sequences in the coding region, 6 GUC sequences in the 5'-untranslated region, and 3 GUC sequences in the 3'-untranslated region. We observed that one GUC sequence is located at codon 179, 6 amino acids upstream from amino acid codon 185, one of the important sites for substrate preference (14-16), and another at codon 196, 11 amino acids downstream from the same site. We reasoned that, if we specifically cleaved the mRNA at a crucial site, the resulting fragments would be nonfunctional, even if the fragments happened to be translated into polypeptides. Accordingly, we designed two hammerhead ribozymes for these two MDR1 mRNA loci and examined their biological activity.

MATERIALS AND METHODS

Cell Lines. The parent MOLT-3 human acute lymphoblastic leukemia cell line (17) and a multidrug-resistant subline MOLT-3/TMQ800 (800-fold TMQ resistant) (18) were maintained in RPMI-1640 medium containing 10% fetal bovine serum and were fed twice a week with fresh medium. MOLT-3/TMQ800 cells were also exposed to TMQ at the concentration of 3 × 10^-6 M for 4 days every 2 weeks. MOLT-3/TMQ800 cells were shown to have P-glycoprotein overexpression and a phenotype identical with P-glycoprotein-mediated MDR. The MDR phenotype in MOLT-3/TMQ800 cells was stable in a TMQ-free medium for at least 6 months. Transfection experiments were carried out after cells were grown in a drug-free medium for 2 weeks, and all experiments with transfected cells were performed within 3 months after transfection.

Ribozyme Synthesis, the Creation of Substrate RNA, and Determination of Cleavage Activity in a Cell-Free System. Human MDR1 gene exons and design of MDR1 ribozymes are illustrated in Fig. 1. Production of ribozymes as well as MDR1 RNA substrate has been described elsewhere (19). Briefly, the 179 MDR1 ribozyme was produced from two synthetic oligodeoxynucleotides, one containing a bacteriophage T7 RNA polymerase promoter, 5'-ATTAATACGACTATAGTTGCCATTCTTATG-3', and the other 5'-AGATGATGTTTCGTCCTCACGGACTCATCACGT-3'. The primers were mixed to form a hemiduplex, and PCR amplification was performed. The 196 MDR1 ribozyme was similarly produced from one primer, 5'-ATTATACGACTATAGTTGCCATTCTTATG-3', and the other 5'-TTTTCAGTTTCGTCCACGGACTCATCACGT-3'. For the production of a ribozyme substrate, total cellular RNA was extracted from MOLT-3/TMQ800 cells. PCR-RT was then performed with oligonucleotide primers from exon 6 (5'-TTCATGCTATAATGCGACAGGAGATA-3') and exon 8 (5'-TTCTTTATCGTAAATGAAGATAGTA-3'). A clear and distinct 275-base pair product was identified in 1.8% agarose gel. The PCR product was ligated directly to pT7Blue T-vector (Novagen, Madison, WI), and after transformation of Escherichia coli, white colonies were selected and screened. Plasmid DNA was prepared by the cetyl-trimethylammonium bromide-DNA precipitation method, and after alkaline denaturation double-stranded DNA sequencing was directly performed. At least 10 clones were sequenced. The clones that produced sense transcript were chosen, and MDR1 RNA substrate was then transcribed from plasmid templates that contained T7 RNA polymerase promoter. RNAs of size 329 bases were obtained and used for cleavage reaction in a cell-free system (19).

In order to ascertain whether the biological effects obtained against the MDR subtype were due to the cleavage reaction, we also created a disabled 196 MDR1 ribozyme, and its cleavage activity in a cell-free system was evaluated. Since the residues indicated by outlined letters in Fig. 2 are known to be important sites for substrate preference (14-16), and another at codon 196, 11 amino acids downstream from the same site. We reasoned that, if we specifically cleaved the mRNA at a crucial site, the resulting fragments would be nonfunctional, even if the fragments happened to be translated into polypeptides. Accordingly, we designed two hammerhead ribozymes for these two MDR1 mRNA loci and examined their biological activity.
Expression of the 196 MDR1 Ribozyme in Mammalian Cells. As described below, the 196 MDR1 ribozyme was found to be more active than the 179 one in a cell-free system. Because of this, subsequent transfection studies were done only with the 196 MDR1 ribozyme.

The 196 MDR1 ribozyme used for transfection study was designed differently from that used in the cell-free system. Two single-stranded oligodeoxyribonucleotides were synthesized such that the 45-base pair ribozyme contained flanking SalI and HindIII restriction sites on both ends and were phosphorylated by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). 5'-pTAGACGTGCTTTCCATTCGAGTAGCTCCGAGACAAGAACTGAA-3', and 5'-pAGCTTCATCCGTCTTCACCCATCGATGATGAGTCCGTGAGGACGAAACTGAA-3' were synthesized according to Haseloff and Gerlach model (13). Codon 185 is one of the substrate preference sites.

RESULTS

Two hammerhead ribozymes synthesized were designated 179 and 196 MDR1 ribozymes based on the position of the codon number of GUC sequence. In order to ascertain whether the DNA sequence of ribozymes designed corresponded with the flanking substrate sequence, the cDNA sequence of the MDR1 gene from MOLT-3/TMQN(1000X) cells was determined. No mutation was found along the cleavage sites for either of the ribozymes (data not shown), indicating that the ribozymes designed could work efficiently.

Cleavage reactions of the MDR1 substrate by 179 and 196 MDR1 ribozymes in the cell-free system are illustrated in Fig. 3. The cleavage reaction was dependent on time, molar ratio of ribozyme:substrate, and magnesium concentration. Comparison of the activity of the two ribozymes showed that the 196 MDR1 ribozyme was more active than the other. The 196 MDR1 ribozyme was cloned into the mammalian expression vector (pH8APr-1-neo), and MOLT-3/TMQN(1000) cells were transfected by electroporation. Forty stable transfecants were selected with G418. All of the subclones grew at a rate virtually identical (population doubling time 22h) with those of the parent MOLT-3 and MOLT-3/TMQN(1000) cells. The VCR sensitivities of the parent MOLT-3, MOLT-3/TMQN(1000) cells, and the transfecants cells are shown in Fig. 4. Clone E, which was representative of 15 clones, was found to be most affected among all clones tested, and the drug sensitivity increased from nearly 700-fold resistance to 20- to 30-fold. Clone D was representative of 18 clones, and clone C was representative of 7 clones. Expression of the ribozyme was demonstrated in the total RNA isolated from the individual clones by the RT-PCR assay. Although the RT-PCR assay was not meant to be quantitative, the increases of ribozyme expression tended to parallel the decrease of MDR1 mRNA expression and P-glycoprotein amount, as well as the degrees of reversal of VCR resistance (Fig. 5). In Northern blots neither MDR1 RNA fragment cleaved by the ribozyme, nor could any low molecular weight species be detected in clones D and E, presumably due to rapid degradation of the ribozyme-cleaved RNA by cellular nucleases.
Fig. 3. Cleavage of MDR1 RNA substrate (329 bases) by 179 and 196 MDR1 ribozymes to 2 cleavage products. A-C, 196 MDR1 ribozyme produced a 178-base 5' fragment (5'F) and 151-base 3' fragment (3'F). D-F, 179 MDR1 ribozyme produced 128-base 5' fragment (5'F) and 201-base 3' fragment (3'F). The substrate was labeled with [α-32P]CTP (specific activity, 800 Ci/mmol; DuPont, Boston, MA) for the in vitro transcription procedure. Ribozymes were not labeled. A, reaction time versus activity profiles of the 196 MDR1 ribozyme. MDR1 RNA substrate and 1/4 molar amount of 196 MDR1 ribozyme were incubated for the indicated time in 50 mM Tris-HCl (pH 7.5) at 37°C in the presence of 10 mM MgCl2. B, cleavage reaction with increasing proportions of the 196 MDR1 ribozyme. Ribozyme:substrate RNA ratio is shown on a molar basis. The reactions were run for 3 h in the presence of 10 mM MgCl2. C, Mg2+ concentration versus cleavage activity. Equimolar amounts of 196 MDR1 ribozyme and substrate were incubated with various concentrations of MgCl2 for 3 h. D, reaction time versus activity profiles of 179 MDR1 ribozyme. Equimolar amounts of ribozyme and substrate were incubated for the indicated time in the presence of 10 mM MgCl2. E, cleavage reaction with increasing proportions of the 179 MDR1 ribozyme. The reactions were run for 12 h in the presence of 10 mM MgCl2. F, Mg2+ concentration versus cleavage activity. Equimolar amounts of 179 MDR1 ribozyme and substrate were incubated with various concentrations of MgCl2 for 12 h.

Fig. 4. Reversal of drug resistance. Dose effect curves: A, MOLT-3; B, MOLT-3/TMQ800; C, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone C); D, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone D); E, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone E); F, MOLT-3/TMQ800 transfected with disabled 196 MDR1 ribozyme. Each point, mean of 3 independent triplicate experiments. All experimental values were within 15% of the mean.

Fig. 5. Analysis of ribozyme expression, Northern blot of MDR1 mRNA, and Western blot of P-glycoprotein. A, RT-PCR products hybridized with the normal ribozyme core probe (specific activity, 800 Ci/mmol; DuPont, Boston, MA) under the high-stringency condition. Under the low-stringency condition, the probe hybridized with not only the normal ribozyme but also the disabled ribozyme (see Fig. 6). B, Northern blot of MDR1 expression. C, Northern blot of GAPDH expression as an internal standard. D, Western blot of P-glycoprotein. Lane 1, MOLT-3/TMQ800; lane 2, MOLT-3/TMQ800 transfected with disabled 196 MDR1 ribozyme; lane 3, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone C); lane 4, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone D); lane 5, MOLT-3/TMQ800 transfected with 196 MDR1 ribozyme (clone E).

Fig. 6. Analysis of ribozyme expression in MOLT-3/TMQ800 cells. Among these, 4 clones were examined for the expression of ribozyme. They had grossly the same degree of expression as clone E (Fig. 6). These data show that the observed reversal of VCR resistance was due to the cleavage activity of the ribozyme and not to the potential antisense effect. The parent MOLT-3 cells were transfected with 196 MDR1 ribozyme, and all 10 stable transfectants analyzed were found to have virtually identical growth rates and VCR sensitivities as MOLT-3 cells (data not shown). Since MOLT-3 cells have no MDR1 expression by Northern blot analysis, MDR1 expression in MOLT-3 cells transfected with 196 MDR1 ribozyme was not performed.

DISCUSSION

In the present study, we created two hammerhead ribozymes designed to cleave the GUC sequence of the codon 179 or 196 in exon 7 of MDR1 mRNA gene in an MDR subline of MOLT-3 human acute lymphoblastic leukemia cell line. Both of the ribozymes cleaved MDR1 RNA, in trans, at specific sites under physiological conditions in vitro: the 196 MDR1 ribozyme produced more efficient cleavage than the 179 MDR1 ribozyme. The cleavage reaction was rapid, and the cleavage product was recognizable within 5 min. The ribozyme used appeared stable for at least 12 h. We found that the optimal Mg2+ concentration was 10 mM or more, but Mg2+ concentrations higher than 10 mM were not tested because intracellular Mg2+ was known to be approximately 0.5 mM (24). MDR1-positive MOLT-3/TMQ800 cells transfected with 196 MDR1 ribozyme became VCR sensitive concomitantly with the decreases of MDR1 expression and the amount of P-glycoprotein. In contrast, disabled 196 MDR1 ribozyme was capable of neither specific cleavage in vitro nor decreasing MDR1 expression in transfectant cells.

Our study provides several useful lessons for the use of ribozyme. First, we found that it is difficult to predict its biological activity. The reasons that 196 MDR1 ribozyme was more active than the 179 MDR1 ribozyme were.

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ribozyme are unclear. It is possible that codon 196 was more exposed on the surface of the mRNA molecule than the 179 site in 3-dimensional structure, and the 196 \( MDR_1 \) ribozyme had easier access to the target sequence. Alternatively, because the 197 \( MDR_1 \) ribozyme contained 5'-GGACU-3' and 5'-AGUCC-3' sequences that could form an unfavorable secondary structure, the ribozyme might not have correctly formed a base pair with the target RNA. In such a case, it could not have acted as an efficient RNA endonuclease. Koizumi et al. (25) have shown that the secondary structure of a ribozyme affects the catalytic activity.

Second, the transfectant cells had various degrees of drug sensitivity. Although ribozyme is known to be reutilized within a cell for a cleavage reaction (26), this feature did not appear to be operative advantageously in a whole cell system. Since efficacy of the ribozyme is dependent on the ribozyme:substrate ratio \textit{in vitro}, a similar phenomenon might have existed within a cell: there were probably not enough ribozyme molecules to cleave \( MDR_1 \) mRNA when cells were constantly overproducing the mRNA. For effective reversal of \( MDR \) phenotype, attempts should be made to transduce as much \( MDR_1 \) ribozyme as possible. To this end, we have initiated studies to use retrovirus vectors as a more effective means of transfer of ribozyme.

Third, the maximal reversal that we accomplished was 20- to 35-fold, and no complete reversal occurred. It is of note that the cell line we used was a highly drug-resistant one. Therefore, it is possible that there were not enough ribozymes transduced within the cell to cleave overproduced \( MDR_1 \) mRNA completely. It is expected that, if \( MDR \) cells with lesser resistance, as commonly observed in clinical samples, were used, complete reversal could occur. Another possibility is that the intracellular Mg\(^{2+}\) concentration is suboptimal for complete cleavage of the target mRNA, even though other divalent cations, \( e.g. \), Mn\(^{2+}\) and Zn\(^{2+}\), may participate in the reaction. It should also be mentioned that the cell lines used were naturally selected and showed 2 independent phenotypes, overproduction of dihydrofolate reductase and P-glycoprotein. Multifactorial resistance is a well-known entity (27). It is possible that the MOLT-3/TMQ80 cells also have mechanisms of resistance other than the \( MDR_1 \) mRNA overexpression. In order to answer this question studies are in progress to produce transfectant cells expressing cloned \( MDR_1 \) CDNA.

We created \( MDR_1 \) ribozyme with a therapeutic intent. This approach may, however, also be used as a laboratory tool for the elucidation of the relationship between mRNA expression and phenotypic characteristics. Ribozymes have been utilized for this purpose to study the expression of oncogenes and viruses (26, 28).

Since ribozymes have antisense sequences against the target, questions can be raised whether ribozyme acts as an antisense rather than a catalytic RNA endonuclease in a transduced cell. The lack of biological effects in the \( MDR \) cells transduced with the disabled ribozyme indicated that an antisense effect was minimal, if it ever existed. For an effective use of ribozyme for the reversal of \( MDR \) in animals and man, additional hurdles must be crossed. First, since P-glycoprotein is known to be present in certain normal tissues and organs (29), \( MDR_1 \) ribozyme should be transduced to target tumor cells only. Second, a system must be developed such that entire tumor cells in the host can be transduced. For this purpose vectors containing tissue-specific enhancer and/or promoter need to be developed. For example, in the case of T-cell leukemia, direct \( i.e. \) injection of retrovirus containing T-cell-specific promoter and \( MDR_1 \) ribozyme might be efficient and relatively tumor cell specific. Since most hematopoietic stem cells which express P-glycoprotein physiologically (30) are in the G\(_0\) phase, they can be protected from infection by retrovirus. All these problems should be solved as issues of drug delivery.

\( MDR_1 \) RNA levels are usually elevated in intrinsically drug-resistant human tumors. \( MDR_1 \) RNA levels are also increased in certain cancers at relapse after chemotherapy (31). In the treatment of acute nonlymphoblastic leukemia, complete remission rates were significantly lower and survival shorter in P-glycoprotein-positive than in P-glycoprotein-negative patients (32). These data indicate that the \( MDR_1 \) expression contributes to \( MDR \) phenotype in many human cancers and leukemias. Development of a means for specific reversal of P-glycoprotein-mediated \( MDR \) is urgently needed.

In spite of the aforementioned anticipated problems to be solved before clinical use, a new specific means to reverse P-glycoprotein-mediated \( MDR \) phenotype is now available. Because of the direct impact of \( MDR \) on the success of chemotherapy and because of the importance of \( MDR_1 \) overexpression on the prognosis of cancer patients, further investigations in this line of work seem warranted.

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**REFERENCES**

22. Kobayashi, H., Takemura, Y., and Ohnuma T. Relationship between tumor cell density


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