Sensitization and apoptosis augmentation of K562/ADM cells by anti-multidrug resistance gene peptide nucleic acid and antisense oligodeoxyribonucleotide[1]

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KEY WORDS peptide nucleic acids; antisense oligodeoxyribonucleotides; MDR genes; P-glycoprotein; multiple drug resistance; apoptosis; leukemia; doxorubicin

ABSTRACT

AIM: To investigate the reversal effect and apoptosis enhancement of peptide nucleic acid (PNA) and antisense oligodeoxyribonucleotide (ASODN) targeted to multidrug resistance gene (*mdr*1) on human multidrug resistant leukemia K562/ADM cells. **METHODS:** A 15-mer PNA and the same sequence of ASODN, complementary to the 5' end of the AUG initiator codon-containing region of *mdr*1 messenger RNA (MDR1-PNA, MDR1-ASODN), were designed and synthesized. Proliferation and sensitivity to adriamycin of K562/ADM cells treated with MDR1-PNA- and MDR1-ASODN were analyzed with a MTT colorimetric assay. Apoptotic morphologies, P-glycoprotein (P-gp) expression, intracellular adriamycin accumulation, and cell cycle were measured. **RESULTS:** MDR1-PNA 1 to 10 μmol/L and MDR1-ASODN 2 to 20 μmol/L alone had no inhibitory effects on the proliferation of K562/ADM cells, but significantly inhibited the growth of K562/ADM cells cultured in adriamycin-containing medium. After treatment with MDR1-PNA and MDR1-ASODN, intracellular adriamycin accumulation in K562/ADM cells increased greatly and P-gp synthesis was strikingly reduced. The resistance to adriamycin of the drug-resistant cells was partly reversed and the cells were induced to apoptosis by adriamycin. The reversal efficacy of MDR1-PNA was 3.1-fold higher than that of the same sequence of MDR-ASODN, but neither MDR1-PNA nor MDR1-ASODN could completely block the *mdr*1/P-gp expression. **CONCLUSION:** Sequence-special PNA targeted to *mdr*1 gene more effectively than the same sequence of MDR1-ASODN inhibited the expression of P-glycoprotein to overcome the drug-resistance.

INTRODUCTION

The multidrug resistance (MDR) of leukemia cells is closely related with the overexpression of P-glycoprotein (P-gp or P-170) encoded by the multidrug resistance 1 (*mdr*1) gene. P-gp is an ATP-dependent drug pump, which confers drug resistance by the active efflux of chemotherapeutic agents from the cells against a concentration gradient. In addition to its ability to efflux toxins, P-gp also inhibits apoptosis induced by a wide array of cell death stimuli relying on activation of intra-

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cellular caspases for full function[1,2]. Antisense strategy is a sequence-selective and -specific inhibiting way for gene expression, in which antisense DNA or antisense RNA has been developed to selectively inhibit or block the expression of target genes, and is an accurate method for blocking the synthesis of a special protein[3].

Peptide nucleic acid (PNA) is a DNA analogue in which the entire negatively charged sugar-phosphate backbone of polynucleotide is replaced by an uncharged polyamide backbone consisting of achiral *N*-(2-aminoethyl) glycine units. Each unit is linked to a purine or pyrimidine base to create the specific sequence required for hybridization to the targeted polynucleotide. PNA have been shown to bind single- and double-stranded DNA and RNA with high affinity and specificity, the absence of a negatively charged backbone facilitates PNA invasion of the DNA double helix to form a stable PNA-DNA hybrid with mismatch discrimination. In the cellular environment, PNA-DNA (-RNA) hybrids are more stable compared with their homologues DNA-DNA or DNA-RNA, and PNA binding to supercoiled DNA is stronger than that to linear DNA and favored in transcriptionally active chromatin. Moreover, the distinctive/unique structure of PNA makes them highly resistant to both nucleases and proteases, and stable in the body fluid and living cells. There are numerous advantages of PNA in anti-gene or antisense applications to down-regulate transcription or translation in living cells[4-6]. In this study, we investigated the comparative effectiveness of a 15-mer PNA and the same sequence of phosphorothioate antisense oligodeoxyribonucleotide (ASODN) to *mdr*1 gene (MDR1-PNA, MDR1-ASODN) on recovery of drug sensitivity and apoptosis in P-gp-overexpressed human multidrug-resistant leukemia K562/ADM cells.

MATERIALS AND METHODS

Reagents MTT and propidium iodide (PI) were purchased from Sigma Chemical Co (St Louis, MO, USA). A phycoerthrin (PE)-conjugated monoclonal anti-P-gp antibody UIC_2 and PE-conjugated nonspecific murine IgG_{2a} were purchased from Coulter-Immunotech Co (Coulter, Margency, France). Adriamycin was obtained from Meiji Pharmaceutical Co Ltd (Tokyo, Japan).

Peptide nucleic acid (PNA) and oligodeoxyribonucleotide (ODN) oligomers PNA and ODN used in this work is complementary to *mdr*1 mRNA location -9–+6, including the AUG start codon[7]. A 15-mer PNA (H-ATC CAT CCC GAC CTC- Lys-NH_2) was synthe-sized and purified by Applied Biosystems (Framingham, MA, USA). The 15-mer fully phosphorioate ODN as the same sequence of the PNA were synthesized by Shanghai Sangon Biological Engineering Service Co Ltd (Shanghai). Both PNA and ODN were dissolved in double distilled water (ddH_2O) to make stock solution, and diluted to working solution before use.

Leukemia cells Adriamycin-selected and P-gp$^+$ multidrug-resistant human leukemia cell K562/ADM[8] and its parental K562 cells were cultured and passaged in our laboratory. All cells were grown in RPMI-1640 medium (Gibco BRL, Grand island, NY, USA) supplemented with 15 % heat-inactivated fetal calf serum (Hyclone, Logan, Utah, USA), 2 mmol/L *L*-glutamine, and antibiotics in a humidified atmosphere at 37 °C in 5 % CO_2. Adriamycin 5.2 μmol/L was added regularly in K562/ADM culture medium to maintain the drug resistance. K562/ADM cells were incubated in adriamycin-free medium for over 1 week and used for experiment.

Detection of growth inhibition of K562/ADM cells by PNA and ASODN Logarithmically growing K562/ADM cells (2×10^8 cells/L) were exposed to various concentrations of MDR1-PNA (1.0 to 10 μmol/L) and MDR1-ASODN (2.0 to 20 μmol/L) up to 96 h, and distributed into 96-well flat-bottom microtiter plate (NUNC, Nunclon, Nagle, Denmark). After these treatments, the cell proliferation was determined with a MTT assay as previously described[9,10].

Measurement of sensitivity of K562/ADM cells to adriamycin K562/ADM cells were grown in 96-well microtiter plates (NUNC, Nunclon, Nagle, Denmark) and administered with MDR1-PNA 5 μmol/L or MDR1-ASODN 10 μmol/L and adriamycin 4.3 to 86.2 μmol/L for 96 h, 1/2 amount of MDR1-ASODN was repeatedly added every 24 h to the cell suspension (Simultaneous treatment); or K562/ADM cells were pro-treated with MDR1-PNA or MDR1-ASODN for 48 h, and then exposed to various concentration of adriamycin for another 48 h (Sequential treatment). Cell growth and viability were detected by the previous MTT assay[9,10]. The 50 % growth-inhibitory concentration (IC_{50}) value for adriamycin at 96 h was determined, and the sensitivity of the cells to adriamycin was calculated by dividing the IC_{50} value of the PNA- or ASODN-treated cells by that of untreated control K562/ADM cells.

Analysis of apoptotic morphology by light and electron microscopes Treated and untreated cells were centrifuged onto sides by cytospin (Zhengzhou, Henan)

and observed with Wright-Giemsa staining by light microscopy. The cytomorphological changes of apoptotic cells were also examined by electron microscopy (JEM-100cx, JEOL Ltd, Tokyo, Japan).

Analysis of cellular apoptosis by flow cytometry After treatment with MDR1-PNA 5 μmol/L or MDR1-ASODN 10 μmol/L plus adriamycin 17.2 μmol/L for 96 h, the flow cytometric evaluation of the apoptotic sub-G1 cell population (apoptotic cells) were performed according to a modification of previously descried method[11]. Briefly, after the administration of MDR1-PNA or MDR1-ASODN combined with adriamycin, the cells were centrifuged, washed in phosphate-buffered saline (PBS), and fixed in cold 70 % ethanol for at least 24 h, and then the cells were stained with PI solution (20 mg/L PI and 20 mg/L RNase in PBS). The sample was read on a Coulter Epics XL flow cytometry (Beckman-Coulter Inc, Fullerton, CA, USA), the percentage of cells in the apoptotic sub-G1 phase was calculated using Multicycle software (Phoenix Flow System, San Diego, CA, USA).

Flow cytometric determination of P-glycoprotein expression K562/ADM cells were treated with MDR1-ASODN 10 and 20 μmol/L or MDR1-PNA 5 and 10 μmol/L for 96 h; and to examine the persistence of P-gp suppression by PNA and ASODN, the treated cells were washed to withdraw the PNA and ASODN, and then continuously cultured in PNA- or ASODN-free medium for 1 to 5 d. The collected cells were incubated with PE anti-P-gp antibody (UIC_2) or non-specific IgG_{2a} in the dark at 4 °C for 30 min, the P-gp positive percentage and mean fluorescence intensity (MFI) of the sample were analyzed by flow cytometry (Coulter Epics XL, Beckman-Coulter Inc, Fullerton, CA, USA)[9,12].

Observation of intracellular adriamycin distribution and content After administration with MDR1-PNA of MDR1-ASODN for 96 h, the K562/ADM cells were incubated with adriamycin (51.7 μmol/L) for 1 h, aliquots of cells were withdrawn, washed with ice-cold PBS twice, ice-bathed, and confocal fluorescence microscopy determination was performed immediately by a Leica TCS SP2 Confocal Laser Scanning Microscopy (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) to reveal the intracellular content and overall distribution pattern of adriamycin[6,13].

RESULTS

Effects of MDR1-PNA and MDR1-ODN on proliferation of K562/ADM cells Both MDR1-PNA and MDR1-ODN did not inhibit the growth and viability of K562/ADM cells. Only a slight decline in cell growth was seen after exposure to higher concentration (>20 μmol/L) of ASODN.

MDR1-PNA and MDR1-ASODN enhanced sensitivity of K562/ADM cells to adriamycin and adriamycin-induced apoptosis The IC_{50} values of adriamycin for K562/ADM cell and its parental cell K562 were (75.0±7.2) μmol/L and (0.7±0.2) μmol/L respectively, and the resistance degree of K562/ADM cells to adriamycin was 106-fold higher than that of K562 cells. K562/ADM cells were co-incubated with MDR1-PNA (5 μmol/L) or MDR1-ASODN (10 μmol/L) plus different concentration of adriamycin for 96 h, the resistance of the cells to adriamycin was partly reverted, and the drug sensitivity of the cells increased by 4.0- and 2.6-fold respectively. Taken into account the used concentration and the achieved effect together, the efficacy of MDR1-PNA was about 3.1-fold higher compared to that of the MDR1-ASODN. There was no difference between simultaneous treatment and sequential treatment (Tab 1).

Tab 1. Recovery of sensitivity of K562/ADM cells to adriamycin by MDR1-PNA and MDR1-ASODN. *n*=3. Mean±SD. $^cP<0.01$ *vs* adriamycin group.

Agents	Adriamycin IC_{50}/μmol·L^{-1}	Potency
Adriamycin	75±7	1.0
MDR1-PNA+adriamycin		
Simultaneous treatment	19±9^c	4.0
Sequential treatment	20±9^c	3.8
MDR1-ASODN+adriamycin		
Simultaneous treatment	29±6^c	2.6
Sequential treatment	34±9^c	2.2

The potency was calculated by dividing the IC_{50} value of adriamycin by that of MDR1-PNA or MDR1-ASODN+ adriamycin.

Treatment of K562/ADM cells with MDR1-PNA or MDR1-ASODN enhanced the sensitivity of drug-resistant cells to adriamycin-induced apoptosis. The treated cells showed cell shrinkage, chromatin condensation, margination, nuclear fragmentation, apoptotic bodies, and intact cell membrane and organelles, the typical cytomorphological features of apoptotic cells.

After K562/ADM cells were co-administered with MDR1-PNA or MDR1-ASODN and adriamycin 17.2 μmol/L for 96 h, the percentage of sub-G1 cells containing hypodiloid amounts of DNA determined by FCM increased from adriamycin control 20.6 % to 42.1 % and 31.1 % by the MDR1-PNA plus adriamycin and the MDR1-ASODN plus adriamycin, respectively (Fig 1). These changes clearly indicated that MDR1-PNA and MDR1-ASODN could sensitize the apoptosis of drug-resistant K562/ADM cells induced by chemotherapeutic agents.

MDR1-PNA and MDR1-ASODN down-regulated expression of P-glycoprotein in K562/ADM cells After treatment with MDR1-PNA 5 and 10 μmol/L, the P-gp positivity in K562/ADM cells decreased by 10.6 % and 16.6 % respectively, and the net decrease of MFI value was 86.4 % and 82.9 %. After exposure to MDR1-ASODN 10 μmol/L and 20 μmol/L, the P-gp positive cells decreased from 98.4 % to 92.3 % and 91.7 %, only a net decrease of 6.1 % and 6.7 % respectively, and the net decline of 78.2 % and 80.6 % were achieved in MFI value (Fig 2). The results suggested that MDR1-PNA suppressed the expression of *mdr*1 gene more effectively than MDR1-ASODN and significantly reduced the synthesis of P-gp, neither MDR1-PNA nor MDR1-ASODN could completely block the *mdr*1/P-gp expression.

To determine the persistence of P-gp suppression in MDR1-PNA- and MDR1-ASODN-treated K562/ADM cells, the cells were washed and incubated in the PNA- or ASODN-free medium for various durations. After 96 to 120 h, the P-gp expression in MDR1-ASODN-treated cells returned nearly to the levels of untreated control cells, but MDR1-PNA-treated cells recovered only 60 % to that of untreated cells.

MDR1-PNA and MDR1-ASODN increased adriamycin accumulation in K562/ADM cells Confocal fluorescence microscopy analysis showed that after incubation with adriamycin 51.7 μmol/L for 60 min, in PNA- and ASODN-treated K562/ADM cell bright adrimycin fluorescence was seen in both nucleus and cytosol, however, only very weak fluorescence was

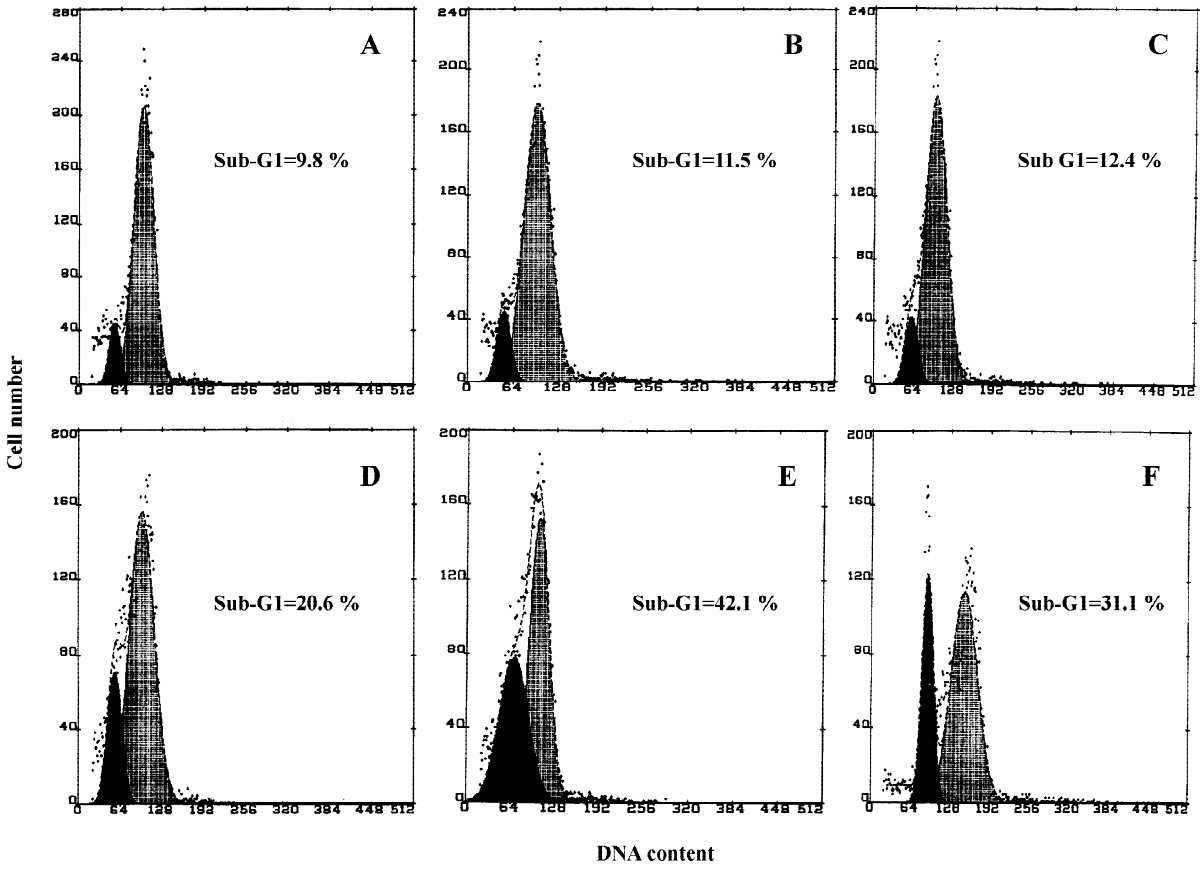

Fig 1. Apoptotic sub-G1 cell population in K562/ADM cells treated by MDR1-PNA, MDR1-ASODN alone or in combination with adriamycin. A: control; B: PNA 5 μmol/L; C: MDR1-ASODN 10 μmol/L; D: adriamycin 17.2 μmol/L; E: MDR1-PNA 5 μmol/L+MDR1-adriamycin; F: MDR1-ASODN 10 μmol/L+adriamycin.

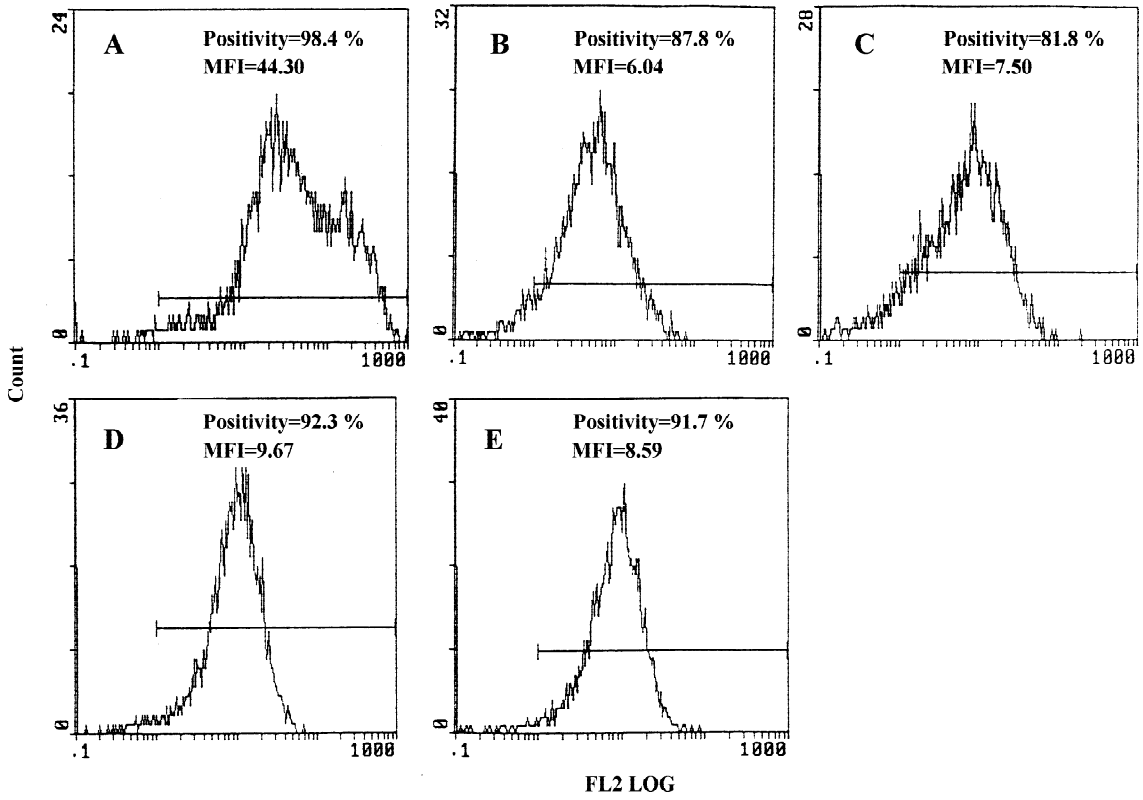

Fig 2. Expression of P-glycoprotein in MDR1-PNA- and MDR1-ASODN-treated K562/ADM cells. A: control; B: MDR1-PNA 5 μmol/L; C: MDR1-PNA 10 μmol/L; D: MDR1-ASODN 10 μmol/L; E: MDR1-ASODN 20 μmol/L.

observed in untreated control K562/ADM cell. The intracellular fluorescence density of adriamycin was also quantified in the final images and on all of the sections of the confocal acquisitions. The distribution and content of adriamycin in PNA- and ASODN-treated cells were markedly different from those in the control cells (Fig 3). These changes may stem from the inhibition of *mdr*1/P-gp expression and the decrease of drug extrusion from the cells by MDR1-PNA and MDR1-ASODN.

DISCUSSION

Antisense ODN targeted to the *mdr*1 gene have been developed to selectively block the expression and function of the gene and its product P-gp. The major obstacles to the use of ASODN are represented by the degradation of nucleases, poor ability to penetrate the cell membrane and nucleus, low affinity and specificity to target genes, and the low ability of inhibiting or blocking the gene expression[7,14-15]. Attempts to improve ODN characteristics have led to development of several derivatives carrying methylphosphonate, methylimino, phosphoramidate, and other internucleotide

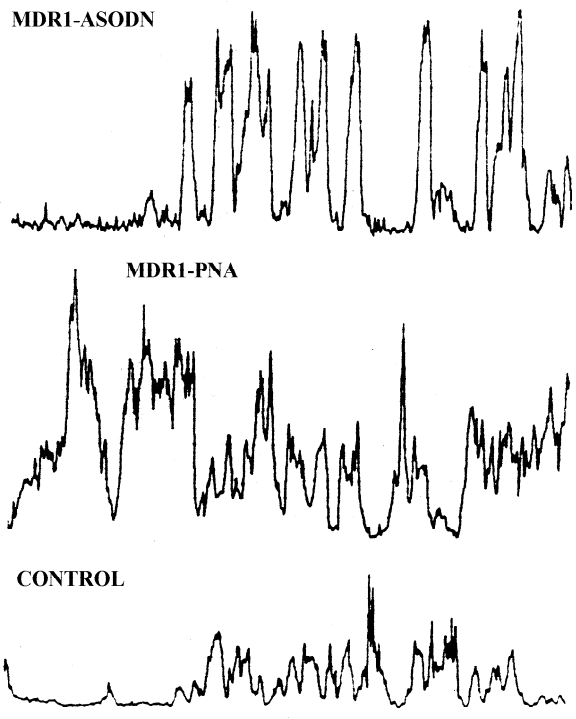

Fig 3. Changes of accumulation and distribution pattern of adriamycin in MDR1-PNA- and MDR1-ASODN-treated K562/ADM cell analyzed by confocal microscope.

linkages. Phosphoramidates of ODN increase intracellular uptake and biological stability, compared with unmodified ODN,but phosphorothioate ODN often reduce the affinity for RNA and lead to nonspecific biological activities not related with their antisense activity[14,16]. PNA actually is a chimera of ' peptide' and nucleic acid bases, which is resistant to degradation of nucleases and proteases, and binds the special sequence of DNA or RNA with high affinity to form PNA-DNA, PNA-RNA, $(PNA)_2$-RNA, $(PNA)_2$-DNA or $(PNA)_2$-dsDNA hybrids, to inhibit transcription/translation of target genes[4-6,16,17].

The 5' -untranslated region (5' -UTR) and the AUG initiation site of mRNA efficiently control the start and efficiency of protein synthesis. In this study, we represent, for the first time, the comparative effects of antisense PNA and phosphorioate ODN, complementary to the AUG region located in 5' -UTR of *mdr*1 mRNA, on the expression of mdr1 gene in living cells. MDR1-PNA and MDR1-ASODN effectively inhibited the expression of *mdr*1 gene in drug-resistant K562/ADM cells to reduce the synthesis of P-gp, the intracellular accumulation of adriamycin increased, the drug resistance of the resistant cells to adriamycin was reversed partly, and adriamycin-induced apoptosis of K562/ADM cells was augmented. The recovery of drug sensitivity and suppression of mdr1/P-gp expression by MDR1-PNA were 3.1-fold much stronger compared with those by the same-sequence MDR1-ASODN in the similar environment. Our data also showed that both MDR1-PNA and MDR1-ASODN achieved limited effects in inhibition of *mdr*1/P-gp expression, and failed to reach relevant blocking of P-gp synthesis. With withdraw of the PNA or ASODN, the P-gp expression recovered gradually, but the recovery degree of the MDR1-PNA-treated cells was much slower than that of the MDR1-ASODN-treated ones.

The gene expression-inhibitory efficacy of MDR1-PNA and MDR1-ASODN achieved from this study is inferior to that reported by other authors[6-7,15-17]. In addition to the complexity of the MDR mechanisms in drug-resistant leukemia cells, this may also be explained as results of difference in selected target sites, poor cellular uptake of large molecular oligomers without other modification, *etc*. Wagner[18] reported that oligonucleotides complexed with cationic liposome localized preferentially in the cell nucleus, whereas oligonucleotides added alone localized mainly in the cytoplasm. Accordingly liposome-encapsulated oligonucleotides seemed to be effective for increasing the cellular uptake and prolonging the action in the nucleus. The PNA terminal position linked to the special vectors were shown a successful delivery to the nucleus of living cells[5,6]. Therefore, in order to increase the efficacy of anti-gene and antisense strategy in overcoming the drug resistance of leukemia mediated by the drug-resistance related genes, it is important in selection of more effective target sites of the candidate genes, and the application of chemical and physical modification to increase the cell uptake and cell-specific targeting. It is also beneficial to try the new intervening models, such as the combination of multiple targets for single gene, combination of anti-gene and antisense strategies for single gene or multiple genes, and combing the anti-gene or antisense methods with other anti-cancer therapies.

In summary, our results show that anti-*mdr*1 PNA down-regulates the *mdr*1/P-gp expression more effectively than ASODN and enhances the sensitivity of the MDR cells to chemotherapeutic drugs to overcome drug-resistance and apoptosis-inhibition mediated by the overexpression of P-gp in multidrug-resistant leukemia cells. It is worth further investigation for the potent significance and potential application of anti-gene or antisense PNA in intervening the multiple drug resistance of leukemia or solid tumors and in treatment of chemotherapeutic agent-resistant acute or chronic leukemia due to overexpression of *mdr*1 gene-encoded P-glycoprotein combined with conventional anticancer drugs.

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