

Expression of multi drug resistance (MDR1) gene in human promyelocytic leukemia cell line selected with vincristine

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ABSTRACT

MDR1 mediated drug resistance is one of the main problems for some cancer chemotherapies. Vincristine (VCR) is an effective drug used in clinical treatment of acute leukemias. The concentration of VCR was stepwise increased (1-50 nM) in the cell culture media of HL60 cells (AML cell line). The viability of cells was determined and the expression of MDR1 gene was studied by using reverse transcriptase-polymerase chain reaction (RT-PCR). In sensitive HL60 cells MDR1 gene expression was not present. The level of the MDR1 gene expression increased in cells with stepwise increases of VCR concentration. MDR1 gene expression was about six times more in 50 nM VCR resistant cells than in 2 nM VCR applied HL60 cells. The IC₅₀ values in sensitive and resistant HL60 cells were 4 nM and 300 nM, respectively. The results indicated that HL60/VCR cells were 75-fold more resistant to vincristine compared to sensitive HL60 cells. It was observed that the cells that were resistant to high concentrations of VCR express MDR1 gene at high levels.[Turk J Cancer 2005;35(2):88-92].

KEY WORDS:

Multidrug resistance, MDR1, P-glycoprotein, HL60, vincristine, AML, RT-PCR

INTRODUCTION

Chemotherapy has been very effective in the treatment of non-localized cancers. However, upon relapse many cancers become resistant to further chemotherapy, even with drugs that have not been previously used. This phenomenon is termed as multidrug resistance (MDR).

The mechanisms underlying this clinical phenomenon have been studied using in vitro models, leading to characterization of genes capable of conferring resistance to chemotherapeutic drugs. Different mechanisms have been characterized in MDR (1). One of the mechanisms that is known to contribute to this phenomenon is the expression of the ATP-dependent P-glycoprotein. P-glycoprotein is included in a large super family of transport proteins termed, ATP Binding Cassette (ABC) transporters or traffic ATPases (2). Among these genes, multidrug resistance gene (MDR1) has been of particular interest because its overexpression can lead to resistance to drugs such as anthracyclines, vinca alkaloids and podophyllins, which are important in the treatment of leukemia (3). MDR1 gene, located on chromosome 7, encodes for a 170-kDa glycoprotein (P-gp). The broad spectrum of drugs affected by MDR1 has made it an attractive candidate to explain the phenomenon of clinical resistance, whereby leukemia cells or tumor becomes refractory to drugs to which it has never been exposed (4).

Clinical significance of MDR1 gene overexpression in leukemia at the time of diagnosis has suggested that it may be appropriate to include elevated MDR1 gene expression levels as an adverse prognostic factor in acute myelogenous leukemia (AML) (5).

Although drug resistance is intrinsically difficult to study in cancer patients, cell lines developed *in vitro* have provided significant insights on the mechanisms of multidrug resistance. Cell lines, which are highly resistant to a variety of anticancer agents, have been generated by slowly increasing the concentration of a cytotoxic agent in a stepwise fashion (6). This approach selects for spontaneous or drug induced genetic alterations which provide a growth advantage in the selecting agent. A common phenomenon noted in cell lines selected with single, cytotoxic agent was that these lines were often cross resistant to a well-defined spectrum of structurally diverse compounds with distinct cellular targets (7).

MATERIALS AND METHODS

Drugs and Chemicals

RPMI 1640, fetal calf serum (0.45 μ m membrane filtered and heat-inactivated), penicillin (10,000 U/ml), streptomycin (10 mg/ml), L-glutamine (200 mM) and Trypan blue solution (0.05%) were obtained from Biological Industries, Israel. Trizol reagent was obtained from Life Technologies. Moloney Murine Leukemia Reverse-Transcriptase, Taq DNA polymerase, RNase inhibitor, DNA size marker (100-3000 bp) were obtained from Fermentas, USA. The set of deoxynucleotide (dNTP), Isopropanol, Agarose, MOPS formamide and MTT were obtained from Sigma, USA. PBS was obtained from Oxoid, England. Diethylepyrocarbonate (DEP-C) was obtained from Applichem, Germany. Formaldehyde (37%) was obtained from Merck, Germany. Oligo-dT was obtained from Integrated DNA Technology, USA. Chloroform was obtained from Lab Scan Analytical Sciences, Ireland. Twenty-five centimeter square tissue culture flasks were obtained from Corning, USA. Seventy-five centimeter square tissue culture flasks were obtained from TPP, Switzerland. VCR was a gift from Dr. Ali Uğur Ural, Gülhane Military Medical School, Ankara, Turkey.

HL 60 Cell Line

HL60 cells were obtained from Gülhane Military Medical School, Department of Haematology, Ankara, Turkey.

HL-60/VCR, a vincristine-resistant line was developed by stepwise exposure of HL-60 to increasing concentrations of vincristine. The cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 500 U/ml penicillin and 0.5 mg/ml streptomycin in 25 cm² tissue culture flasks. The cells were incubated in CO₂ incubator at 37°C in the presence of 5% CO₂. The medium was refreshed every five days.

Cell Survival (MTT) Assay

2x10⁴ cells were plated in 100 ml of the growth medium in the absence or presence of increasing concentrations of VCR into 96-well plates at 37°C in 5% CO₂ for 24 h and 48 h. The cells were then incubated with 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) (5 mg/ml) at 37°C for 4 h. Then the medium was removed and the converted dye was solubilized with the addition of acidic isopropanol (0.1 N). Plates were examined by using a microplate reader at 570 nm and the concentration of drug that inhibited cell survival by 50% (IC₅₀) was determined from cell survival plots (8).

Isolation of RNA

Total RNA was isolated from 1x10⁷ sensitive HL60 and HL60/VCR cells by using Trizol reagent (consist of guanidium thiocyanate, phenol and sodium citrate). Quantification of RNA was conducted by spectrophotometer. Measuring the absorbance at wavelengths of 260 nm and 280 nm will provide information about protein contamination of RNA. Reading at 260 nm was used to calculate the concentration of nucleic acid in a sample (9). The intactness of total RNA was confirmed by two sharp bands 28S rRNA and 18S rRNA (10). Total RNA was stored at -80°C.

Reverse Transcription

Reverse transcription was carried out for 60 min at 42°C in a total volume of 20 μ l, containing ribonuclease inhibitor (20 U), 0.5 mg oligo-dT, 2.5 mM of each dNTP and 200 units of Moloney Murine Leukemia Virus-reverse transcriptase and a sample of 3 μ g of total cellular RNA. Once the cDNA copy created using the mRNA template, the PCR was conducted immediately or the cDNA was stored at -20°C until required for analyses.

Polymerase Chain Reaction

Two sets of primers were used in all reactions to obtain amplification of an endogenous control, β -2-microglobulin (120 bases) and a specific target gene of interest, MDR1 gene (258 bases). Following an initial denaturation step of 94 °C for 5 minutes, 35 cycles of PCR amplification were performed, each consisting of a denaturation step of 94 °C for 30 seconds, annealing at 62 °C for 45 seconds and extension at 72 °C for 1 min. At the end of the 35 cycles, a 5-minute extension phase at 72 °C was included to provide complete synthesis. The amplified fragments were visualized by running on 2% agarose gel at 90 V for 1 hour and then by ethidium bromide staining.

Primers were obtained from IDT, USA. The PCR primers used to amplify MDR1 gene were 21/20 nucleotide long oligonucleotides. The sequence of sense strand primer was 5'TACAGTGGAAATTGGTGCTGGG and the sequence antisense strand primer was 5'CCCAGTGAAAAATGTTGCCA. Nucleotides were chosen to avoid any co-amplification of MDR3 gene (11). For the amplification of the β -2-microglobulin 20-mer oligonucleotides were used. The sequence sense strand primer was 5'CTTACTGAAGAATGGAGAGAGA and the sequence antisense strand primer was 5'CTTACATGTCTCTATCCCCTT (11).

RESULTS AND DISCUSSION

HL60 Cells and Drug Application

The concentration of VCR was gradually increased. For this purpose, cells split into several culture flasks containing different concentrations of Vincristine. Cells in lower concentrations of drug can propagate but some of the cells at higher concentrations may die. The flasks where only 50% of the cells die were selected and propagated. Since resistance changes as an exponential manner, the increments of VCR concentration was also accelerated. Percentages of viable cells were determined by using Trypan blue method (12).

Before application of Vincristine, HL60 cells were grown for a few weeks to adapt HL60 cells to laboratory conditions and to optimize the growth. Then different drug concentrations (from 1 nM to 50 nM) were applied to HL60 cells in a stepwise fashion. 10 nM and 50 nM VCR concen-

trations seemed to be critical for HL60 cells. At these drug concentrations longer periods were needed for growth and adaptation of cells. HL60 cells adapted to each drug concentration around 3-4 weeks. However cells were able to adapt to 10 nM and 50 nM VCR concentrations only after the 6-week incubation. Adaptation of HL60 cells to applied drug concentration was tested by Trypan blue method. By using this technique, percentages of viable and dead cells were determined under microscope. The next higher concentration of drug was applied to the cell line when 90% of cells were alive.

Expression of MDR1 mRNA

The intactness of total RNA was confirmed by two sharp bands which are 28S rRNA and 18S rRNA separated on denaturing agarose gels and visualized by ethidium bromide (EtBr) staining under UV light (10).

The target MDR1 and β -2-microglobulin as reference gene were amplified by PCR. The PCR product of MDR1 gene as 258 bp fragment and the PCR product of β -2-microglobulin gene as 120 bp fragment are seen in figure 1.

The level of target gene expression was determined by the intensities of the resulting PCR product bands on the gel. The ratio of band intensities of MDR1 gene and β -2-microglobulin gene was determined at each drug concentration. This ratio is an important parameter for the expression pattern of MDR1 gene.

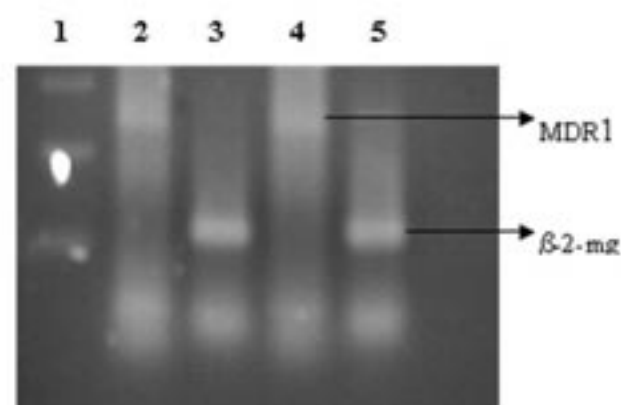


Fig 1. Agarose Gel Electrophoresis of PCR products from 40 nM and 50 nM VCR applied HL60 Cells. Lane 1. DNA Ladder, Lane 2. MDR1 expression in 40 nM VCR applied cells, Lane 3. β -2-Microglobulin expression in 40 nM VCR applied cells, Lane 4. MDR1 in 50 nM VCR applied cells, Lane 5. β -2-Microglobulin in 50 nM VCR applied cells

MTT Cell Viability Assay

The IC_{50} value at each drug concentration was determined from the cell survival plots and results are given in figure 4. IC_{50} value for sensitive cells was found to be 4 nM. Whereas for HL60/VCR, it was 300 nM (Figure 2). In this study 50 nM of VCR was applied as the highest concentration and 75 times more resistant cells were obtained compared to sensitive HL60 cells (Figure 3).

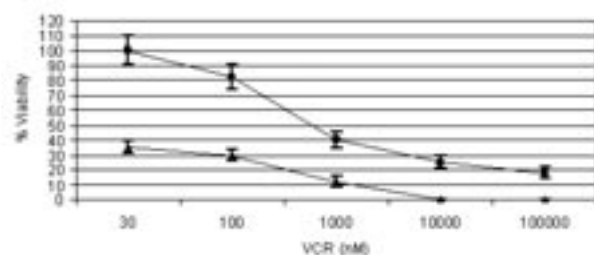


Fig 2. Cytotoxic effects of vincristine (VCR) on HL60 cell. VCR resistant (HL60/VCR) (circles) and sensitive (HL60/wt) (triangles) cell lines were cultured with increasing concentration of VCR for 24 hours. The data depict the mean \pm SD of 3 separate experiments. The cytotoxic effect was most prominent at increasing concentrations of VCR on HL60/wt cells whereas the resistance was impressive on HL60/VCR cells ($p < 0.001$ at 100 nM of VCR)

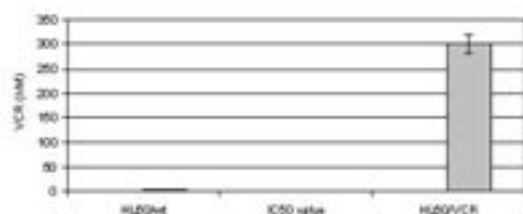


Fig 3. IC_{50} values of VCR for HL60/wt and HL60/VCR cells

DISCUSSION

In the current study, leukemic cell line, HL60 was used as an in vitro model for the investigation of drug resistance in a human hematological malignancy (AML). Drug resistant cell line was established by stepwise increasing concentrations of VCR in the cell culture.

It can be clearly seen from figures 4 and 5, the size of HL60 cells became smaller and the cell surface became rough as VCR concentration was increased. The shape of HL60 cells changed with the application of VCR. Cells

may protect themselves against the toxic drug by becoming less permeable and they may express different sets of proteins on the cell membrane as a defense mechanism.

In PCR, mRNA of β -2-microglobulin was used as an internal control. It has been shown that β -2-microglobulin gene expression was continuous and was not changed by exposure to VCR (12). The level of target gene expression is reflected in the ratio between the intensities of the MDR1 and β -2-microglobulin PCR product bands on the gel. This ratio is used in comparisons of the expression levels of MDR1 gene.

PCR products of MDR1 gene and β -2-microglobulin gene from 40 nM and 50 nM VCR resistant HL60 cells are shown in figure 1. Normally HL60 cells do not express P-glycoprotein (13). It was observed that HL60

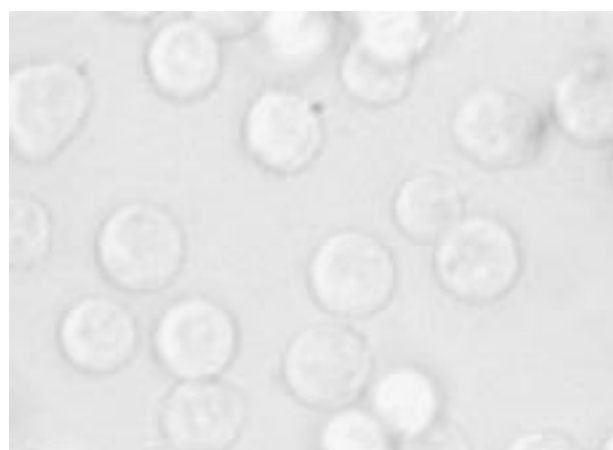


Fig 4. Micrograph of HL60 cells before application of VCR

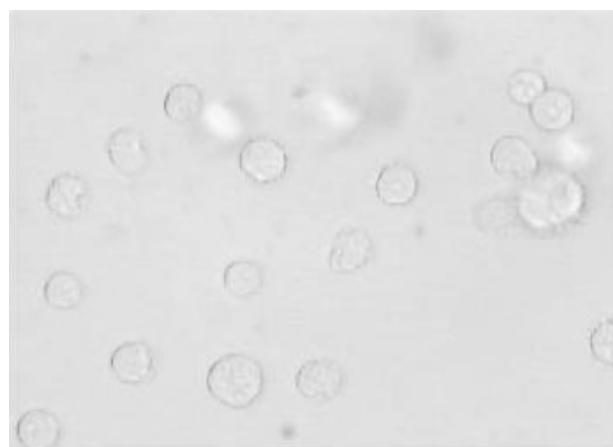


Fig 5. Micrograph of 50 nM VCR applied HL60 cells

cells started to express MDR1 gene in 2 nM vincristine. In the sensitive and 1 nM drug applied HL60 cells, MDR1 gene expression was not present. In 50 nM VCR applied HL60 cells (Figure 1), MDR1 gene expression was 6 times more compared to 2 nM VCR applied HL60 cells by densitometric analyses. The results strongly suggest that HL60 cells can survive at higher concentrations of VCR and this resistance can be explained by overexpression of the MDR1 gene.

The IC_{50} values of VCR in sensitive and resistant HL60 cells were determined. IC_{50} values were found to be 4 nM for sensitive cells and 300 nM for HL60/VCR cells (Figure 2). The results indicated that final HL60/VCR cells gained 75-fold resistance compared to sensitive HL60 cells. These results are comparable to those in literature (13,14).

Measurements of the expression of the MDR1 gene in AML cell line will be helpful in the understanding of drug resistance and predict drug responsiveness in leukemia before or during chemotherapy.

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