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Biology and Modulation of Multidrug Resistance (MDR) in Hematological Malignancies

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Abstract

Drug resistance is one of the most significant impediments in the treatment of hematological malignancies. There have been a number of studies on the incidence of P-GP expression in tumor cells or tissues, where detectable level of P-GP has been found in all types of hematological malignancies. P-GP expression and significance in the patients varies widely between reported studies on patients with different ages and different disease types. Some of this validation can be accounted for by the threshold used to consider a sample positive for P-GP. However, *mdr-1* is likely important in determining therapeutic outcome in patients with AML, NHL, and MM, although there is a suggestion of a different "behavior" between adult and childhood AML. In contrast, the significant prognostic association with expression of MRP and LRP is not consistent with disease types and disease stages. Clinical trials of modulation of MDR have been limited by following major factors. One is inability of achieving adequate blood levels of the modulator to reverse MDR, and the other is presence of other resistance mechanisms in addition to P-GP. The fact that P-GP modulators alter the pharmacokinetics of anti-cancer drugs can potentially increase toxicities if the dose of anticancer drugs is not appropriately reduced. Recently, MDR modulators such as valspodar have demonstrated substantial inhibition of P-GP. In this presentation, a number of characteristics in VCR-resistant cells are reported. We demonstrate that acquisition of MDR or recovery from MDR phenotypes differ in one cell type to another, a marked correlation between P-GP and susuceptibility to oxygen radicals, and altered gene expression of cell membrane antigen and apoptosis cascade genes. The efficacy of immunotherapies depends on the altered or unchanged target molecules of MDR cells. Thus, immunotherapies or reversal agents that aim at these substances in tumor cells should be useful to overcome MDR phenotypes.

1. Introduction

1.1. Multidrug Resistance (MDR) Phenotypes

The multidrug resistance (MDR) is one of the major impedediment in cancer treatment, which can be occurred by the alterations in drug transport, altered intracellular drug targets, and altered apoptosis mechanisms and metabolic mechanisms. Recently, three proteins that are related with the alteration of drug transport are found. One is the drug resistance protein, permeability-related glycoprotein (P-GP), and the other factor is multidrug-resistance associated protein (MRP). The third one is lung resistance protein (LRP) found subsequently in the cytoplasm of tumor cells showing MDR phenotype.

P170-kd P-GP is encoded by the *mdr-1* gene, locarized at 7q21.1 [1,2], a transmembrane glycoprotein consisting of two domains and upregulation of this protein results in a decreased intracellular concentration of anthracyclins, vinca-alkaloids and epipodophylotoxins. Although the normal physiological function of P-GP remains unknown, P-GP exerts its action to reduce intracellular drug accumulation, which causes MDR as a result of the initial stage of the therapy or by the chemotherapy after relapses. At the cellular level, the function of P-GP has been extensively investigated in

many types of cancer cells. In leukemia patients, cellular drug resistance profiles determined *in vitro* at the time of presentation show a strong correlation with outcome.

MRP1 was identified in cell lines showing typical MDR phenotype without elevated P-GP [3]. P190-kd MRP is encoded by the *mrp* gene, located at 16p13.1. MRP possesses the characteristic structural motifs of P-GP, and like P-GP is a member of the ABC-transporter superfamily. The amino acid homology between P-GP and MRP1 is 15%. Although a transmembrane protein, anti-MRP antibodies stain mainly the intracellular epitopes. The physiological role of MRP1 is unknown, but inside-out plasma membrane vesicles isolated from MRP1-overexpressing cells show an increased ATP-dependent transport of glutathione S-conjugates and gluconate and sulphate conjugates. Evidence that intact cells require glutathione (GSH) for extrusion of several drugs by MRP1 has been obtained [4]. Like P-GP, MRP1 is involved in altered drug distribution within intracellular components in cytoplasm, leading to concentrations of cytoplasmic drugs at their target sites.

LRP was initially identified in anthracycline-resistant, non-small cell lung cancer cell line which was characterized as an MDR-phenotype but which lacked P-GP expression [5]. The LRP gene was located at chomosome 16p13.2, proximal to the MRP gene on chromosome. LRP is 110-kd and a member of the vault protein family of ribonucleoproteins and it's the major human vault protein accounting for more than 70% of the mass of vault particles. LRP is not an ABC-transporter protein, although it is thought to be involved in transmembrane transporter of various substrates. The main function still needs to be identified, but the main target site of LRP may be intracellular and associated with the transport of drugs into and out of the nucleus since vaults are colocalized in the nuclear membrane and vesicles [6]. Like P-GP and MRP, it has been suggested that LRP in normal tissues plays a role in detoxication process.

1.2. Analytical Methods for MDR Phenotypes

It is difficult to compare *mdr-1* expression in cell lines and clinical samples from different studies because of different detection methods (Immunocytochemistry, flow-cytometry, RNase protection assays, and quantitative PCR), use of different threshold for positivity, use of different internal controles and various monoclonal antibodies, comparison of different expression levels among DNA/RNA/Protein, and differences in methods employed to purify leukemic blasts. *Mdr-1* gene is differently expressed in a variety of normal tissues, particularly along the apical surface of secretary epithelium of the jejunum and colon, proximal tubular epithelium, and the glandular epithelium of the pregnant uterus, in addition to in the adrenal grand, placenta, capillary endothelium of the brain and testis, as well as in hematopoetic precursors and lymphocytes [7,8]. MRP1 has been detected in all human tissues and in every cell type of peripheral blood. Levels of MRP1 are low only in erythrocytes and liver canaliculi.

1.3. Prognostic Significance of MDR Phenotypes in Hematologic Malignancies

In AML, *mdr-1* overexpression at diagnosis is a strong impediment predictor for CR and long-term survival, although there is a suggestion of a different "behavior" between adult and childhood AML [9-11]. In ALL, *mdr-1* expression is of minor importance for prediction of outcome. In AML, MRP1 expression at diagnosis is not correlated with clinical response and survival in most of studies [12]. In ALL, MRP1 expression at diagnosis is not associated with response and long term survival in few studies [13]. The studies on LRP in AML emphasize the importance of the correlation between LRP-expression and anthracycline accumulation and suggest that LRP-expression has a prognostic value at diagnosis [14]. However, there are an equal number of studies where a predictive value in the case of LRP-expression in *de novo* AML cannot be shown [15].

There are a number of studies on the incidence of P-GP expression in non-Hodgikin's lymphoma (NHL); detectable levels of P-GP have been found in all histological types of NHL [16]. P-GP expression has been correlated with drug sensitivity and clinical outcome in many studies of NHL [17]. However, there are some reports indicating no correlation between response and P-GP expression [18]. Thus, it is presently unclear whether MDR expression has a significant impact on the response to therapy in lymphoma.

P-GP expression does not seem to occur in *de novo* multiple myeloma (MM) patients, since myeloma cells at diagnosis neither express elevated levels of *mdr-1* mRNA nor stain with the anti-P-GP antibodies [19]. However, after exposure to vincristine, adriamycine, and dexamethazone (VAD) chemotherapy, the expression of *mdr-1* reaches levels above detectable [20]. MRP is not over-expressed in MM, but LRP is expressed in half of the MM patients, and is associated with a poor response to melphalan at conventional dose [21].

1.4. The Methods of Overcoming of MDR Phenotypes Using P-GP Modulators

Two possible approaches to *mdr-1* reversal by agents can be distinguished. First, some agents such as *mdr-1*-specific anti-sense oligonucleotides and protein kinase C inhibitors such as staurosporin are capable of down-regulating *mdr-1* expression [22,23]. The second one is modulating agents that can restore drug accumulation by competing with cytostatic drugs for P-GP binding sites. These agents include calcium channel blocker, some type of cardiovascular drugs, cyclosporin analogues, and anti-malarias [24]. The function of MRP1 can be blocked by many compounds having a variety of structures. The effect of P-GP modulators on MRP1 were less than their effect on P-GP over-expressing cells. Up to now, clinical intervention studies with *mdr-1* modifying agents

have only been done with AML patients using modified analogue to cyclosporin D, valspodar [25]. Although the results of the few studies with P-GP modifiers in the hematological malignancies are promising, the data are insufficient for recommending the routine use of P-GP modulators to increase disease-free survival in leukemia.

Thus, we fully need to explore the potential of transporter-specific modulators in improving clinical outcome, more knowledge will be needed on the nature, substrate specificity, inhibitory sensitivity and expression of efflux pump responsible for MDR in human cancer.

2. Materials And Methods

2.1. Wild Type Cultured Cell Lines from Leukemia/ Lymphoma Used in This Study

Five cultured cell lines from human leukemia and lymphoma were used in this study. One cell line, K-562 derived from chronic myelogenous leukemia, which expresses both *mdr-1* and *mrp*, and the other 4 cell lines which express only *mrp* were chosen to be made resistant to VCR. The latter 4 cell lines consisted of an acute promyelocytic leukemia cell line, HL-60, a Japanese (non-endemic) Burkitt's lymphoma (BL) cell line, BL-TH, a B-type cell line from immunoblastic lymphoma, A4/fuk, and a T-type chronic lymphocytic leukemia (CLL) cell line, SKW-3. In the studies on testing the efficacy of immunotherapies, Daudi cell line expressing only *mrp* from African BL and its VCR-resistant cell line were used. The cells were maintained in RPMI-1640 supplemented with 10% FBS, 100µg/mL penicillin-G, and 100µg/mL streptomycin at 37°C in humidified 5% CO_2 in air.

2.2. Measuring of Chemosensitivity of Cultured Cells for VCR

To determine the 50% inhibitory concentration (IC50) for VCR, 0.5-2x10^5 cells/mL in 60µL of fresh medium containing various concentrations of drug were cultured in a 96-well round bottom microtest plate. At 4th day of culture, 10µL of 0.15 mg/mL propidium iodide (PI) dissolved in phosphate buffered saline (PBS) containing 1.2% polyoxyethylene octylphenyl ether was added to each well. After 20 min at room temperature, the fluorescence intensity (FI) was measured in MPV-MT2 automated reading system. The FI of test samples was correlated with the cell counts.

2.3. Detection of P-GP and MRP on Cultured Cell Lines

To detect P-GP, 5x10^5 cells in a Fisher tube were incubated for 30 min on nice with MRK-16 or mouse IgG as a negative control. Cells were washed three times with PBS containing 10% FBS, then incubated again with FITC-goat anti-mouse IgG for 30 min on ice. Cells were washed three times and suspended in PBS containing 10% FBS for analysis using a laser flowcytometer.

Because anti-MRP antibody, MRPr1, reacts with an internal epitopes of human MRP1, cell permeabilization was required as a pretreatment step. For this purpose, buffer consisted of PBS containing 2% FBS and 0.1% NaN3 was developed. The 5x10^5 cells were pretreated with 2% formaldehyde in acetone for five seconds. After washing two times with the buffer, the cells were incubated for 30 min on ice with rat monoclonal antibody to human MRP1 or rat serum as a negative control. After washing three times by the buffer, cells were incubated with FITC-conjugated mouse monoclonal anti-rat IgG2a. Cells were washed three times again and suspended in the buffer for analysis using a laser flowcytometer.

2.4. RNA Extraction and Semi-quantitation of Gene Expression by RT-PCR

Total RNA from 1×10^7 cells which had been washed with ice cold PBS was extracted by a one step acid guanidine thiocyanate-phenol-chloroform method using an ISOGEN-LS total RNA purifying kit. Concentration and purity of the RNA were determined spectrophoto-metrically and the samples were stored at -80°C until use.

Reverse transcription (RT) was carried out for 60 min at 37°C in a total volume of 20µL of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 26 units of Ribonuclease inhibitor, 200 units of Moloney Murune Leukemia Virus Reverse Transcriptase RNase H Minus with 100 pmol random primer on each sample of 2µg total cellular RNA. The polymerase chain reaction (PCR) was performed with 50 ng cDNA, 25 pmol of each primer, 1 mM dNTP, 1 unit Ampli*Taq* polymerase in 25µL of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatine) using a GeneAmp PCR system. Electrophoresis was visualized on a Printgraph. The level of mRNA expression was analyzed using NIH image. The expression was shown as the relative level to the intrinsic β *2MG*.

3. Results

3.1. EstabLishment of VCR-Resistant Cell Lines and Their MDR Phenotypes

Five cultured cell lines were made resistant to VCR by stepwise incubation. The final degree of VCR-resistance in a drug-free medium for one week ranged from 36.2- to 112-fold increase as compared to the corresponding wild type cells. The VCR-resistant HL-60 cell line was named as HL-60/VCR, and a 112-fold resistance to VCR compared to wild type cells shows in the cell line name as HL-60/112VCR. The degree of VCR-resistance level of Daudi/VCR was low; 2 times as high as the wild type cells.

In the preliminary RT-PCR experiments using the total RNA from the wild type cell lines, in which the cycle number of PCR went through 35 cycles, only

K-562 was found to weakly express *mdr-1* mRNA. Since the *mdr-1* products increased linearly through 30 cycles, the gene expression was semi-quantitatively evaluated by 28 cycles of PCR. In contrast, the expression of *mrp* and *lrp* was observed in all wild type cell lines. K-562/VCR, HL-60/VCR and BL-TH/VCR increased or acquired the *mdr-1* expression. In contrast, three VCR-resistant cell lines, K-562/VCR, A4/fuk/VCR and SKW-3/VCR increased the *mrp* expression. The increase of *lrp* expression was observed weakly in K-562/VCR and markedly in BL-TH/VCR, but it decreased in SKW-3/VCR. Thus, K-562/VCR increased all genes of *mdr-1, mrp*, and *lrp*, HL-60/VCR increased only *mdr-1*, BL-TH/VCR increased both *mdr-1* and *lrp*, and A4/fuk/VCR and SKW-3/VCR increased only *mrp* gene.

All wild type cells expressed genes of *WT1* and *bcl-2*. An obvious correlation between *WT1* and *mdr-1* gene in cells that increased *mdr-1* was confirmed after attaining the higher level of drug resistance. We also found an obvious correlation between *bcl-2* and *mrp* gene in VCR-resistant cells. However, no genes tested associated with the change of *lrp* in these cells.

3.2. Recovery from MDR Phenotypes in Drug Free Culture

It is not known if malignant cells that have once attained MDR phenotype recover their drug sensitivity during the intermission of therapy and how the MDR-related phenotypes behave during this period. In an attempt to understand how MDR phenotype changes during the intervention of therapy, we followed up the change of MDR phenotypes in 5 types of VCR resistant cell lines after releasing drug *in vitro*. Out of five VCR-resistant cell lines, only one cell line of BL-TH/VCR markedly recovered its sensitivity to VCR after culturing for six months without VCR, although the relative resistance level was still 2-3 times higher than wild type cells. The change of three VCR-resistant cell lines, K-562/VCR, HL-60/VCR and SKW-3/VCR, was very characteristic with increasing once their resistance level at fifth weeks. The resistance level declined thereafter but those levels were kept plausible high after 6 months. One cell line of A4/fuk/VCR increased its resistance level, which declined soon and had been kept low after the drug removal, more than initial resistance level around 6 months of this culture. The change of drug resistance level in K-562/VCR, HL-60/VCR and BL-TH/VCR was paralleled with the expression level of *mdr-1* but not with its protein product of P-GP. In A4/fuk/VCR and SKW-3/VCR, their VCR-resistance was considered to be partially dependent on the expression of *mrp,* but the whole change of drug resistance could not be explained by any of five types of gene expressions tested, *mdr-1*, *mrp*, *lrp*, *wt-1*, and *bcl-2*. These results indicate that the mechanism of MDR phenotype in each cell lines is different and behaves in various manners after the cessation of drugs. Although the *in vitro* results may not be able to be applied directly to the patient's fresh cells, our data indicate that every cell is protected by the different MDR mechanisms and behave differently after the cessation of anti-neoplastic drugs. Thus, the intervention of anti-neoplastic drugs is not enough to recover the drug sensitivity if malignant cells have acquired MDR phenotypes, suggesting the necessity to develop the treatment method which can inhibit MDR mechanisms.

3.3. Increased Resistance to Oxygen Radicals through Altered Cell Membrane Structure in VCR-resistant Cell Lines and Overcoming by P-GP Modulators

Active oxygen radicals injure cell membrane by oxidizing their lipids and some type of antineoplastic agents such as adriamycine, bleomycine, and etoposide exert their efficacy through the cytotoxic activity of oxygen radicals. In an attempt to develop the treatment methods to overcome MDR phenomenon using techniques other than P-GP modulators, we investigated the sensitivity of cell membrane against oxygen radicals which were obtained hypoxanthine-xanthine oxidase system. A marked correlation between P-GP and susceptibility to oxygen radicals was found. In contrast, there was no correlation between MRP and susceptibility to oxygen radicals [26]. These results may suggest a new mechanism of drug resistance in cells expressing P-GP. In addition, we found that cyclosporin A (Cy-A) enhances the cytocidal effect of oxygen radicals. Neither Verapamyl (Ver) nor Dipyridamole (Dip) showed any effect on cytocidal activity of oxygen radicals. Thus, it was suggested that Cy- A works with different mechanisms from those of Ver or Dip, and Cy-A may be useful in reducing the doses of anti-neoplastic agents that exert their activity via oxygen radicals in the clinical treatment [27].

3.4. Overcoming of MDR by Immunotherapies Using Cytocidal Activity of PBL, LAK, and IDEC-C2B8

Because it has become apparent that the cell membrane of tumor cells showing P-GP has altered, we tested the efficacy of immunotherapies such as PBL, LAK, and anti-CD20 chimeric mouse/human monoclonal antibody, IDEC-C2B8, which can be expected to work as different mechanisms from anti-neoplastic drugs, against VCR-resistant cells.

Three of six VCR-resistant cell lines were less susceptible to PBL cytotoxicity compared with wild type cells, whereas the susceptibility was kept in the other three cell lines. Four of six VCR-resistant cell lines were less susceptible to LAK cells as compared with their counterpart wild cells. There was no correlation between susceptibility for PBL cytotoxicity and the expression of HLA class 1 antigen in both wild and VCR-resistant cells. In contrast, ICAM-1 in the two cell lines that showed decreased susceptibility for LAK cytotoxicity disappeared, although that in one cell line increased. IDEC-C2B8 was effective only against B-cell

lines expressing CD20 antigen. One cell line, Daudi/VCR, in which the expression of CD20 had been up-regulated was nearly six times more sensitive to IDEC-C2B8 than wild type cells [28]. Thus, we concluded that the resistance to VCR in some tumor cell lines is associated with modified susceptibility to immunotherapies by the different expression of target molecules from those of wild-type counterparts.

3.5. AltereD Gene Expressions Such as Tumor Suppressor Genes, Oncogenes, and Apoptosis Cascade Genes in Vcr-Resistant Cells

Tumor suppressor genes, oncogenes, and genes on the apoptosis cascade pathway are involved in the basic biological process governing cell death and survival. To access the regulation mechanism of MDR, we investigated expression of genes that had been known as the genes involved in poor prognosis of hematological malignancies. In cells that acquired *mdr-1*, down-regulation of *p53* and up-regulation of *wt-1* were observed. The *wt-1* tumor suppressor gene was originally found in association with the etiology of Wilms' tumor. Recently, it was shown that *wt-1* is expressed in various types of leukemia. Although the significance of the expression of the *wt-1* gene in leukemia is unclear, the poor prognosis and the higher level of *wt-1* gene expression have been linked [29]. Using cell lines showing higher level of VCR-resistance, we confirmed this to be definitive, suggesting *wt-1* gene as a promising prognostic factor for the leukemia. These results may provide a plausible diagnostic marker for determination of drug sensitivity in the patients with hematologic malignancies and suggest that *p53* may mediate directly or indirectly the expression of *mdr-1* via *wt-1* in VCR-resistant cells. Because the *wt-1* mediates the expression of many genes, there may be a possibility that *wt-1* participates in the regulation of the *mdr-1* despite no direct evidences for this speculation [30].

In contrast, no constant change was observed in cells that over-expressed *mrp*. Although the change in the expression level of *cyclin D1* and *p16* accompanied the development of VCR resistance, the mRNA of *rb*, *c-myc*, and *n-my*c showed no correlation with the degree of VCR-resistance or the level of *mdr-1* expression.

As a new finding, an association between *bcl-2* and *mrp* gene was found. The *bcl-2* gene is well known because of its up-regulation leading to the prevention of apoptosis [31]. The *bcl-2* is also reported to be one of the prognostic factors in leukemia [32,33]. However, the association of both genes in drug resistant cells has not been known. Thus, the up-regulation of *bcl-2* gene also can be a plausible marker for chemoresistance in leukemia cells. Some genes on the apoptosis cascade such as *TNF-R*, *Fas-R*, *Caspase-3*, and *Bax-α* seem to be involved in the development of MDR caused by *mdr-1*.

4. Conclusions

In spite of major advances of cancer chemotherapy, drug resistance is one of the most significant challenges facing clinicians who treat hematological malignancies. There have been a number of studies on the incidence of P-GP expression in ALL, AML, NHL, and MM, where detectable level of P-GP has been found in all types of hematological malignancies. P-GP expression and significance in the patients varies widely between reported studies reporting on patients with different ages and different disease types. Some of this validation can be accounted for by the threshold used to consider a sample positive for P-GP. However, *mdr-1* is likely important in determining therapeutic outcome in patients with AML, NHL, and MM. The significant prognostic association with expression of MRP and LRP is not consistent with disease types and disease stages.

Clinical trials of modulation of MDR have been limited by two major factors: inability of achieving adequate blood levels of the modulator to reverse MDR in patients, and the presence of other resistance mechanisms in addition to P-GP. A third factor is the fact that P-GP modulators alter the pharmacokinetics of anti-cancer drugs by delaying their elimination: this can potentially increase toxicities if the dose of anticancer drugs is not appropriately reduced. However, because it has been demonstrated that MDR modulators such as valspodar demonstrated substantial inhibition of P-GP, reversal agents that only inhibit P-GP in tumor cells and do not influence the pharmacokinetics of cytotoxicic agents should be developed. Thus, we fully need to explore the potential of transporter-specific modulators in improving clinical outcome, more knowledge will be needed on the nature, substrate specificity, inhibitory sensitivity and expression of efflux pump responsible for MDR in human cancer.

On the other hand, as shown in this presentation, acquisition of MDR phenotypes by the stepwise incubation and alteration of MDR phenotypes after removal of drug from culture system differ in one cell type to another. On the basis of the altered or unchanged surface antigens and apoptosis cascade genes, immunotherapies or inhibitors aiming at these substances may be useful.

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