MDR Genes in Leukemia

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Introduction

Multidrug resistance (MDR) describes the phenomenon of simultaneous resistance to unrelated drugs expressed by drug-resistant cells when compared to their drug-sensitive counterparts. Studies with cell lines have revealed that multidrug resistance can develop rapidly in culture. This raises the possibility that similar MDR tumor cells can arise spontaneously in human cancers, providing an explanation for the frequently observed non-response to chemotherapy in many advanced malignancies. This possibility has resulted in intensive research in the past two decades to investigate the genetic and molecular basis of MDR. Different cellular mechanisms are capable of mediating MDR. These include reduced drug accumulation involving the P-glycoprotein (MDR1) gene(1) and the multidrug resistance-associated protein (MRP) gene,(2) drug detoxification involving the glutathione-S-transferase genes,(3) altered drug targets involving topoisomerase II(4) and alterations in drug-induced apoptosis involving genes in the Bcl-2 pathway.(5) The focus of this presentation is on the MDR phenotype associated with the P-glycoprotein (Pgp) and MRP genes. These two genes are members of a superfamily of ATP-binding membrane associated transport proteins (ABC transporters) widely distributed in all kingdoms of life.(1)

A major challenge is to determine what roles cellular MDR mechanisms play in a patient's response to chemotherapy. This is not a simple question to answer. When Pgp was used as a molecular marker for the detection of MDR tumor cells in clinical samples, it was observed in a broad range of human tumor cells.(6,7,8,9) In some instances of childhood malignancies, in multiple myeloma, and in malignant leukemia, clinically relevant correlations between Pgp expression and the results of chemotherapeutic treatment have been observed.(10,11,12) Such correlative evidence, however, does not constitute sufficient proof that the Pgp MDR mechanism plays a limiting role in a patient's response to chemotherapy. Since Pgp is differentially expressed in normal tissues it is possible that in some instances Pgp is simply a marker of differentiation and may not be causative of chemotherapy resistance. Only when we are able to specifically inactivate the MDR mechanism in tumor cells and to demonstrate a concomitant durable clinical response upon treatment with chemotherapy can we be confident that the MDR mechanism characterised in vitro plays a causative role in the response of patients to cancer chemotherapy

Human leukemias are among the most extensively studied tumors for drug-resistance mechanisms. The ease of obtaining biopsies, the possibility of culturing leukemic cells and the ability to manipulate biopsies for various functional assays make these malignancies ideal for investigating the role of MDR genes at the clinical level. Much of the material covered in this lecture will involve Pgp since this is the best characterised of the MDR mechanisms. Many of the principles and concepts covered with Pgp will apply to MRP and other MDR mechanisms.

Molecular Biology of Pgp and MRP

Both Pgp and MRP belong to the superfamily of ABC Transporters.[1] These genes have been cloned and transfected into drug sensitive cells, with the resulting recipient cells displaying an MDR phenotype. Such studies provide definitive evidence that Pgp and MRP are causative of MDR. In the Pgp family, there are two genes in human (MDR1 and MDR2/3) and three genes in rodents. These genes are closely linked on the chromosome and selection of cell lines for high multidrug resistance often results in the amplification of the MDR genes plus some flanking genes. Co-amplification of non-MDR genes in resistant cells could add to the complexity of the MDR phenotype. The P-glycoprotein gene is located on chromosome 7 while the MRP gene is located on chromosome 16. Both Pgp and MRP are large membrane proteins greater than 1200 amino acids in length. The structure of both proteins contains multi-spanning transmembrane segments and two highly conserved ATP-binding domains. It has been hypothesised that the P-glycoprotein gene evolved from an ancient gene duplication event since the N- and C-terminal halves show internal homology to each other. Each half contains six predicted transmembrane segments and an ATP-binding domain. The predicted topologic structure of MRP, however, is different, with eight predicted transmembrane segments in the N-terminal half and four in the C-terminal half. Within the ABC transporter family, the MRP gene and the Pgp gene are evolutionarily very distant. The MRP gene is more closely related to the cystic fibrosis gene CFTR. In fact, the P-glycoprotein gene is evolutionarily related more to the bacterial hemolysin B gene than to the mammalian MRP gene. Thus, it is not possible to predict substrate specificity nor whether a particular ABC transporter is capable of an MDR phenotype from structural information alone.

Although Pgp and MRP both cause multidrug resistance, their patterns of resistance are different (see Table 1). It is interesting to note that a number of lipophilic compounds able to reverse the Pgp-mediated MDR have been identified (these include verapamil (VP), cyclosporin A (CSA), calmodulin inhibitors, progesterone and many others).[13] CSA and VP are widely used to block Pgp function but they appear less effective against the MRP-mediated MDR. Mutational analyses of Pgp have indicated that point mutations within or close to predicted transmembrane segments can affect drug specificity and sometimes response to reversing agents. The concept that the transmembrane domain of ABC transporters dictates substrate specificity has been corroborated in mutational studies in the bacteria hemolysin transport system, where single point mutations in the transmembrane domain of hemolysin B can dramatically alter substrate specificity.[14]

Table 1. P-glycoprotein and MRP-associated MDR phenotypes.

	Relative Drug Resistance			
Cell line	ADM	VCR	VP-16	COL
H69/LX4	85	~1000	88	80
H69 AR	54	35	40	~5100

Relative resistance is calculated as a ratio of the drug concentration required to inhibit cell growth in the resistant line divided by that required for the parental drug sensitive line. H69/LX4 and H69AR are human small cell lung cancer lines independently selected for resistance to adriamycin from the parental H69 line. H69/LX4 over-expresses P-glycoprotein and contains an amplified MDR1 gene. H69 AR possesses an amplified MRP gene and its P-glycoprotein expression is not increased. ADM = adriamycin; VCR = vincristine; VP-16 = etoposide; COL = colchicine. (Table adapted from Childs and Ling, 1994.)

Although molecular genetic studies clearly implicate Pgp and MRP as causative of MDR, how these proteins recognise and transport a broad spectrum of drugs remains a mystery. There have been speculations that Pgp acts via indirect mechanisms such as altering intracellular pH or modifying membrane potential.[15] Alternatively, it has been speculated that drugs transported by Pgp need to be modified by some ligand recognised by the transporter. Only by purifying Pgp and functionally reconstituting it in liposomes were we able to determine that Pgp alone is sufficient for the transport of multiple drugs.[15] Moreover, it was possible to demonstrate that energy for transport is derived from magnesium ATP and an intrinsic Pgp ATPase. This series of studies is important since it demonstrated that Pgp can recognise a number of different drugs directly and that it does not require modification of the drugs for recognition. This implies that Pgp has a broad substrate specificity. Further detailed investigation of this substrate recognition may provide insights as to the range of substrates potentially recognised by Pgp. Efforts are currently ongoing to purify and reconstitute the MRP protein. It would be very interesting to determine whether MRP can recognise drugs directly similar to Pgp or whether additional modification such as conjugation of the drug is required. A number of studies have implicated MRP as a "GS-X pump," suggesting that it recognises glutathione conjugated compounds.[16,17,18] Other studies suggest that MRP recognises and transports compounds such as daunorubicin directly. All these studies were undertaken with whole cells or with membrane vesicles. Only with a purified functional system will it be possible to sort out the complexities of this system.

Normal Function of Pgp and MRP

Delineating the normal role of Pgp and MRP continues to be an area of active investigation. As mentioned earlier, Pgp is comprised of a small family of genes in mammalian cells. The MDR1 gene causes drug resistance, while the MDR2/3 genes appear not to cause drug resistance. By analogy to the rodent system, the MDR2/3 gene is likely to be involved in the transport of phosphatidylcholine in the bile canaliculi of liver. This conclusion was drawn from homologous recombination inactivation in knockout mice of the mdr2 gene.[19] A similar knockout of the mdr1a gene in mice demonstrated that this gene is involved in the blood-brain barrier and in the transport of drugs such as in the intestinal tract.[20,21] The knockout studies of the P-glycoprotein genes in mice provide invaluable insights as to the function of these genes in the whole animal. They demonstrate that the different Pgp isoforms have unique functions. The discovery that the mouse mdr2 gene product transports phospholipid was an unexpected surprise. The

MDR1 gene associated with drug resistance functions to protect the organism against a broad range of xenobiotics. This has implications for efforts to block the MDR1 gene function chemically, for example, by the use of chemosensitizing agents in order to improve chemotherapy in malignancies that express Pgp. The pharmacokinetics of anticancer drugs may be affected when Pgp function is blocked. This may result in unwanted toxic side effects; alternatively, it may open up pharmacologic sanctuaries normally protected by Pgp, for example, in the brain or other organs for treatment with certain types of therapy.

A similar knockout study with the MRP gene has not yet been reported. MRP appears to be more widely and uniformly distributed in the tissues of animals. It would be of interest to determine if an MRP knockout results in the survival of the animal and whether or not this could affect drug distribution.

The Use of Pgp and MRP as Molecular Markers of MDR in Leukemia

The fact that transfection of the MDR1 and the MRP gene results in multidrug resistance indicates that the products of these genes may be used as molecular markers for the presence of MDR cells in patients. In general, the level of mRNA expressed correlates with the level of protein although there have been exceptions. In order to determine if expression of these genes correlates with non-response in chemotherapy, a methodology that reliably detects the presence of these genes or gene products in biopsy samples needs to be employed. Several considerations need to be undertaken. First, because the MDR phenotype is dependent on the level of the gene product expressed, detection must be at least semi-quantitative. Second, it must be highly sensitive and specific. Third, ideally the methodology should allow detection of these gene products in archival material in order to undertake retrospective studies. Fourth, the ability to detect expression of these genes at the single cell level has the advantage that expression of these genes in normal cells versus tumor cells can be differentiated. Table 2 lists the strengths and weaknesses of some of the approaches used for the detection of Pgp in clinical samples. In leukemias, the use of flow cytometry along with specific antibodies has been a powerful tool for detecting the gene products of MDR mechanisms. It has the advantage of sampling many cells and is able to discriminate between normal and tumor cells. In addition, for the Pgp and MRP mechanisms there is the further possibility of employing a functional assay to determine if an altered drug accumulation mechanism can be detected. In this manner, independent lines of evidence can confirm the presence of the MDR phenotype.
Table 2. Detection of P-glycoprotein in clinical samples.

Desired Properties

1. Applicable to formalin-fixed paraffin-embedded material (e,f)
2. Distinguish between normal and tumor tissues (e,f)
3. High sensitivity (c)
4. Quantitative (a,b,d)

Techniques

a. RNA slot blot

b. Northern blot
c. RT-PCR
d. Immunoblot
e. Immunohistochemistry
f. RNA in situ hybridization

To date, dozens of studies in leukemias using different approaches either to detect the MDR1 gene expression at the nucleic acid level or at the gene product level using antibodies have been undertaken.[8,22] The results from these studies have not always agreed with respect to the proportion of patients considered to have a significant level of MDR1 tumor cells. This is due in part to a lack of a universal standard for quantitating expression.[22] It is difficult to compare one study against another because of the different methodologies used for the detection of MDR genes. Within individual studies, however, a pattern has emerged. Usually, when P-glycoprotein or MRP is detected at presentation, the level of expression is usually higher in subsequent relapses. On occasion, Pgp is not detected at presentation but is detectable upon subsequent relapses. Thus, the opportunity to obtain sequential samples from a single patient during the course of the disease is extremely valuable when attempting to correlate the expression of any MDR gene with the clinical course. Regardless of which technique is used for the molecular detection of an MDR mechanism, thresholds for clinically significant levels of expression must be established by appropriate clinical designs and studies.

A number of studies of acute leukemias have supported the concept that the presence of Pgp in blast cells correlates with poor prognosis for therapy. For example, Ma et al[23] were the first to demonstrate that Pgp may be over-expressed in AML in two patients. Their leukemic cells were negative for Pgp at diagnosis, but one patient became positive at first relapse and the other on recovering from second induction chemotherapy. Two large studies of 61 and 63 patients, respectively, that correlate MDR1 expression with response to treatment have been examined.[24,25] A significant association between low remission rates/short remission duration with expression of MDR1 as measured by RNA slot blot hybridization analysis was observed. Subsequently, many studies have supported these early observations.[22]

As noted above, a number of compounds are able to reverse the Pgp-mediated MDR phenotype. VP and CSA are able to sensitize the Pgp-mediated MDR phenotype in vitro and have been used at the clinical level with the intention of applying such agents to improve clinical efficacy. Results using flow cytometry to detect Pgp function indicate that CSA and CP stimulate accumulation of anthracycline into blast cells of AML patients expressing Pgp. For example, Ross et al[26] have found that CSA causes enhancement in intracellular accumulation of daunorubicin in blast cells of approximately half of AML patients by the time of disease presentation, suggesting the possibility of Pgp-related MDR phenotype being expressed in those patients. A phase I/II clinical trial using CSA in combination with a modified regimen of araC and daunorubicin in poor-risk AML patients obtained a complete remission rate of 62% and immediate remission duration of 13 months.(27) Such a result has not been seen with similar protocols in high-risk patients. These findings support two studies of multiple myeloma[10,28] in which the use of VP or CSA in Pgp-positive patients appears to induce response using the same

drug combination (VAD) to which the patient's disease have previously not responded. Pgp negative patients were not similarly affected. Taken together, the above results indicate that Pgp expression correlates with advanced disease in AML and that the presence of Pgp positive tumor cells may be an important factor limiting response in such patients. Such studies are exciting but they raise further significant questions. For example, it would be important to determine if patients who fail in the presence of CSA or VP acquire other MDR mechanisms. Along with this is the question of the mechanism of resistance associated with likely a large proportion of AML patients who are non-responsive to chemotherapy and who do not appear to express a significant level of Pgp nor MRP. It may be speculated that other members of the ABC-transporter family not yet identified are involved in such cases.

It is now increasingly evident that more than one MDR phenotype may be expressed in an MDR cell.[29,30] In earlier works, selection for high drug resistance in cell lines results in gene amplification and, usually, a single MDR mechanism (for example, Pgp over-expression or MRP over-expression) predominates. Such a situation is not the norm in clinical samples. It is possible that even a lower expression of Pgp or MRP or other MDR mechanism may be significant. If this is the case, it would be important to determine the repertoire of MDR mechanisms that are relevant for any particular malignancy. As noted above, we do not as yet have a clear idea as to what level of expression of an MDR mechanism is clinically relevant. Such research is most easily carried out in leukemias.

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