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Biochemical, Genetic, and Metabolic Adaptations of Tumor Cells That Express the Typical Multidrug-Resistance Phenotype. Reversion by New Therapies

Loris G. Baggetto[1]

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Among the genetic and metabolic alterations that cancer cells undergo, several allow their survival under extreme environmental conditions. The resulting aberrant metabolism is compatible with tumor progression at the expenses of high energy needs, especially for maintaining high division rates. When treated with chemotherapeutic drugs many cancer cells take advantage of their ability to develop a resistance phenotype, as part of an adaptative mechanism. Two main actors of this multidrug phenotype (MDR) are represented by the P-glycoprotein and by the more recently discovered multidrug-resistance associated protein (MRP), two membrane proteins of the ABC superfamily of transporters that can extrude chemotherapeutic drugs under an ATP-dependent mechanism. We will briefly review the major metabolic aberrations that several cancers develop, followed by the molecular, genetic, structural, and functional aspects related mainly to P-glycoprotein, with a concern for the regulation of *mdr* gene expression. We will point out the role that membrane cholesterol may play in the MDR phenotype, relate this phenotype to bioenergetic considerations, and review the ways of modulating it by the use of new therapeutic approaches.

KEY WORDS: Antisense; bioenergetics; gene therapy; membrane; metabolism; multidrug resistance; P-glycoprotein; ribozyme; transcriptional regulation.

HISTORICAL BASES FOR AN UNUSUAL METABOLISM

Upon studying rapidly growing cancer cells, Warburg noticed in 1926 their high glycolysis rate. He proposed a hypothesis according to which the observed high glycolysis was due to impaired respiratory capacities of these rapidly-growing, "glycolytic" tumor cells. These data led to the discovery and understanding of a highly deviated general metabolism that transformed tumor cells into energy-dissipating entities.

Later in 1948, Lepage raised the question of an intensive nonoxidative decarboxylation of pyruvate in glycolytic tumor homogenates, which was not to be accounted for by lactate formation, and the products of which could not be clearly identified (Groth *et al.*, 1952). In 1977, Greenhouse and Lehninger showed, by using isotopic methods, that 90% of the glycolytic pyruvate was reduced to lactate by intact glycolytic tumor cells; only a small quantity of the remaining 10% of pyruvate underwent oxidation through the Krebs cycle, especially in the presence of glutamine considered as the major respiratory substrate in cancer cells through a way that is called glutaminolysis (Kovacevic, 1971). In this pathway, glutaminase is a progression-linked enzyme (Linder-Horowitz *et al.*, 1969) the localization of which has recently been assigned to the matrix side of the inner mitochondrial membrane of Ehrlich ascites tumor cells, the enzyme being anchored to the membrane (Aledo *et al.*, 1997).

Several supplementary elements arose to sustain the hypothesis of an abnormal pyruvate oxidation in tumor cells. Even though both transport and utilization

[1] IBCP, UPR 412 CNRS, 7, Passage du Vercors, F-69367 Lyon Cedex 07, France.

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of pyruvate are not altered in tumor mitochondria (Paradies *et al.*, 1983), the pathway of added pyruvate to tumor mitochondria differs from that of internally-generated pyruvate due to the progression-linked, intramitochondrial NAD(P)$^+$-malic enzyme, which is present in these tumors (Moreadith and Lehninger, 1984a). Such a progression-linked enzyme may furnish acetyl-CoA units to feed the Krebs cycle. Upon its entry into mitochondria, pyruvate undergoes an oxidative decarboxylation catalyzed by the pyruvate dehydrogenase complex. The small fraction of coenzyme A produced from glycolytic pyruvate undergoes oxidation through the tumoral Krebs cycle, the first part of which has been found to be truncated at the citrate level (Parlo and Coleman, 1984). Citrate is preferentially exported toward the cytoplasm where it serves for the synthesis of cholesterol, a membrane steroid the level of which is highly increased in many cancer cell types (Lavietes and Coleman, 1980). Other anomalies of pyruvate metabolism in cancer cells concern the adaptive detoxification from aldehydes through acetoin synthesis (Baggetto and Lehninger, 1987a) since the intracellular distribution of aliphatic aldehyde dehydrogenases differs from that of normal cells (Lindahll, 1979). Moreover, acetoin turned out to exert several control effects on tumor energetic metabolism such as a competitive inhibition with pyruvate on tumor pyruvate dehydrogenase (Baggetto and Lehninger, 1987b).

High glycolytic activity ensures the survival and the migration of tumor cells in hypoxic areas (Epner *et al.*, 1993).

However, several cases have been reported where a high glycolytic rate was not linked to cell proliferation: several cell lines are able to grow in a medium with 5 mM galactose or with low glucose supply (0.5 mM) without producing lactate *via* glycolysis (Wice *et al.*, 1981, Lanks, 1987) but by pyruvate oxidation or by conversion of glutamine to lactate (Sauer *et al.*, 1980, Moreadith and Lehninger, 1984a,b, McKeehan, 1982). When those cells are replaced in a medium with a high glucose concentration (5 mM), all phosphometabolites above pyruvate kinase accumulate until the level of fructose 1,6-bisphosphate is high enough to activate pyruvate kinase (Eigenbrodt *et al.*, 1992). Lactate derives thus from glucose and all intermediates of glycolysis between hexokinase and pyruvate kinase increase. Even though pyruvate kinase is activated, this mechanism ensures the supply of phosphometabolites for anabolism (Eigenbrodt *et al.*, 1992). Moreover, since growth factors and oncogene-dependent phosphorylation regulate both glycolysis and phosphometabolite pools, some phosphometabolites or their derived products such as sugar phosphates, AMP, NAD, and NADH may regulate cell proliferation (Eigenbrodt *et al.*, 1992, Oude Weemink *et al.*, 1992; Smith and Merrill, 1995, Hugo *et al.*, 1992). It has been found recently that extracellular AMP inhibits DNA synthesis in MCF-7 cells and stops cell proliferation (Mazurek *et al.*, 1997).

Other metabolic deviations of cancer cells have been reviewed (Pedersen, 1978; Coleman and Lavietes, 1981; Baggetto, 1992), such as their increased rate of glucose transport (Flier *et al.*, 1986) and specific properties of enzymes that regulate glycolysis (Pedersen, 1978). However, the most important cause for the high rate of glycolysis in tumor cells has been evidenced by Pedersen since 1977. Rapidly growing hepatomas do not have efficient enzymatic activities involved in gluconeogenesis (Sweeney *et al.*, 1963). Their hexokinase is of a fetal type and the proportion of the mitochondrial activity with regard to the cellular one is important (40–60%) (Bustamante and Pedersen, 1977). Pedersen's group has shown that the mitochondrial porin was part of a receptor proteic complex for a bound form of hexokinase. This enzyme binding to the outer mitochondrial membrane allows an efficient glucose phosphorylation by ATP originating from mitochondrial oxidative phosphorylations. Hexokinase bound to the outer mitochondrial membrane is functionally coupled to the adenine nucleotide translocase, thus directly feeding the glycolytic system with the glucose-6-phosphate it produces. The latter is used to provide cellular ATP through glycolysis and the tricarboxylic acid cycle. It is also the source of precursors for other important metabolites necessary for tumor cell growth and division. Membrane-bound hexokinase has been demonstrated to be linked to tumor progression (Bustamante *et al.*, 1981).

In such a tumor system, mitochondria-bound hexokinase, together with glycolytic enzymes such as tumor pyruvate kinase, give the glycolytic system a higher competitive capacity with regard to available Pi and ADP in the cell. However, such metabolic deviations cost tumor cells an elevated energetic price.

WHERE A DRUG RESISTANCE FEATURE INVOLVES ANOTHER TUMOR CELL ADAPTATION

The Multidrug Resistance (MDR) Phenotype through Two Major Components

In the hope of fighting cancer, the broad usage of chemotherapeutic drugs has led to the emergence

of the concept of chemoresistance, which represents today one of the main causes of cancer therapy failure.

In the majority of cases of clinical resistance in tumors, two main factors may play a major role. First, pharmacological factors are those that prevent tumor cells from being adequately exposed to the chemotherapeutic agent; they may relate to medication and to intrinsic genetic variations. Second, cellular factors are those that are intrinsic to the tumor cell itself, which has probably developed several resistance mechanisms.

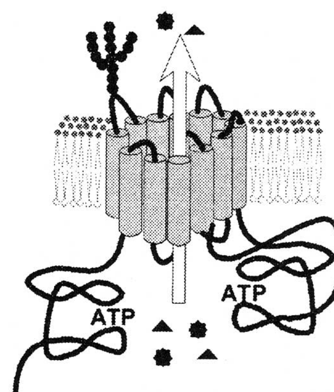

Fig. 1. Functional model of P-glycoprotein.

P-glycoprotein (Pgp)

Multidrug resistance is usually the result of many phenomena that define the multidrug resistance (MDR) phenotype. These phenomena include reduction of the sensitivity of DNA topoisomerase II to cytotoxic drugs, drug metabolism, plasma membrane and intracellular changes, metabolic adaptation, and upregulation of plasma membrane pumps. Most of the latter proteins belong to the ATP-binding cassette (ABC) superfamily of transporters. P-glycoprotein (Pgp), the first that bas been discovered (Juliano and Ling, 1976), is believed to function as an ATP-dependent cytotoxic anticancer drug efflux pump, and is related to the "typical" MDR phenotype. Complete purification of Pgp from different species has recently been achieved by our group which allowed the first approaches to its structural studies (Dong *et al.*, 1996).

P-glycoprotein is the product of the *mdr* gene that belongs to a small family of highly homologous genes (Ueda *et al.*, 1986). These genes can be ranked into two main classes: the first class contains genes that are related to cancer MDR, whereas the second class contains genes for which the function of their product is not yet fully known, except that they are not related to MDR. There are two human genes (*MDR1* and *MDR2,* also called *MDR3*) and three rodent genes (*mdr*1 or *mdr*1b, *mdr*2, and *mdr*3 or *mdr*1a) (Gros *et al.*, 1991; Roninson *et al.*, 1991). Among the two human genes, only *MDR*1 is expressed at a high level in MDR cell lines (Kane *et al.*, 1990) and gene mapping studies have localized human *MDR*1 genes in chromosome 7q21.1. Cancer cell chemoresistance *in vitro* is generally correlated with *MDR*1 gene amplification (Cole *et al.*, 1992).

Human Pgp contains 1280 amino acids for a molecular mass of 140 kDa. A glycosidic moiety is linked to the first extracellular loop of the protein, close to its *N*-terminal end; it does not appear to be involved in the function of the protein (Beck and Cirtain, 1982). The Pgp contains two homologous halves reunited by a linker region. Each one of the two moieties is formed of a hydrophobic *N*-terminal region that contains six transmembrane helices followed by a hydrophilic C-terminal region oriented toward the cytoplasm (Endicott and Ling, 1989). The *C*-terminal region contains one nucleotide binding site (Chen *et al.*, 1986) characterized by the two structural elements A and B (Walker *et al.*, 1982), which define Pgp as a member of the superfamily of the ABC transporters. A three-dimensional model of Pgp (Fig. 1) shows a probable channel through which substances such as anticancer drugs involved in the MDR phenotype are actively transported toward the extracellular medium so that it maintains intracellular drug concentrations below the cytotoxic level (Kirschner *et al.*, 1992). This model has recently received some support from a 2.5-nm-resolution electron microscopy study showing, when viewed from above the membrane plane, a 10-nm-diameter toroidal protein with a 6-fold symmetry bearing a 5-nm central pore. The two 3-nm lobes exposed at the cytoplamsic face of the membrane may stand for the two nucleotide binding domains (Rosenberg *et al.*, 1997). Moreover, the absence of apparent specificity of Pgp for the many drugs it extrudes led to the proposal of a flippase model (Higgins and Gottesman, 1992) that would export only drugs that interact first with the membrane lipid bilayer before binding to Pgp.

Multidrug-Resistance Associated Protein (MRP)

Several non-Pgp proteins involved in the development of the MDR phenotype have recently been described (for a review see Baggetto, 1997). To date,

the only non-Pgp protein that has been confirmed to play a role in the MDR phenotype is the multidrug-resistance-associated protein (MRP) that was initially found as an overexpressed membrane glycoprotein in a multidrug-resistant human lung cancer cell line (Cole *et al.*, 1992). MRP is mainly localized in the plasma membrane (Zaman *et al.*, 1994) and occasionally in the endoplasmic reticulum (Hipfner *et al.*, 1994; Krishnamachary *et al.*, 1994). The gene coding for MRP has been localized in the human chromosome 16p13.1. MRP is a 190-kDa membrane protein that contains eight transmembrane segments followed by a nucleotide binding domain in the first half of the molecule, followed by four transmembrane segments and a second nucleotide binding domain in its second half (Krishnamachary and Center, 1993). Both nucleotide binding domains are in the cytoplasmic side of the cell. This molecule, like Pgp, belongs to the ABC superfamily of transporters and is at the origin of resistance to both natural products and to heavy metal anions (Cole *et al.*, 1994). It too undergoes phosphorylation of serine residues probably by protein kinase C (Ma *et al.*, 1995; Almquist *et al.*, 1995). The molecule is overexpressed in a number of non-P-glycoprotein multidrug-resistant cells and isolates (Barrand *et al.*, 1994).

Biochemical Activity of P-glycoprotein

Because of its two nucleotide-binding domains, Pgp is believed to function as an ATPase pump. Since recently, purification of functional Pgp has been very problematic mainly because of its strong membrane embedding and also probably because of the need for a proper membrane lipidic environment to maintain this biochemical activity. We have recently been able to completely purify a lipid-free Pgp from different tumor types and functionally reconstitute it into liposomes; we could show that this entity was able to catalyze ATP hydrolysis and transport [^{3}H]-vinblastine in an ATP-dependent manner (Dong *et al.*, 1996) (Table I). However, maximal substrate-stimulated ATPase activity is reached in membrane extracts or in partially purified Pgp (Ambudkar *et al.*, 1992; Sharom *et al.*, 1993; Shapiro and Ling, 1994) while a basal ATPase activity (in the absence of drugs) may be due to specific closely-related lipids in the membrane or to endogenous substrates present in the preparations; it may also reflect an "uncoupled"-type of ATPase activity since it has recently been demonstrated that the mutation Gly185→Val185 decreased this basal activity three times (Ramachandra *et al.*, 1996). One can imagine that if a regulatory factor is lost during the purification procedure, the coupling between Pgp ATPase activity and drug transport is no longer present in reconstituted Pgp. ATPase activity of Pgp is stimulated by a number of drugs and chemosensitizers, all related to the MDR phenotype. However, several chemosensitizers such as verapamil and trifluoperazine provide the highest degree of stimulation for reasons that still remain unclear. In the same way, inhibition of ATPase activity is obtained with cyclosporin A (Rao and Scarborough, 1994). Inhibition may occur according to at least two ways: the inhibitor may affect either drug binding or both drug binding and ATPase activity.

*MDR*1 Gene Regulation in Human Cancers

*MDR*1 is commonly expressed in cancers derived from tissues that normally express that gene. However, the phenotype may also appear before or during chemotherapy in tumors originating from tissues that were not expressing *MDR*1. Besides the evident tissue specificity of *MDR*1 expression, the heckling former feature may be explained by disregulation of *MDR*1 during tumor progression and induction of *MDR*1 expression by chemotherapeutic drugs. Since the regulation of the *MDR*1 gene is not yet fully understood, the question of how the MDR phenotype takes place as a tumor cell response and adaptation to environmental stresses is crucial.

Cytogenetics of MDR Amplification

The MDR phenotype developed *in vitro* is due to gene amplification associated to cytogenetic anomalies (Biedler *et al.*, 1988). Several aberrant chromosomal structures have been discovered such as homogeneously stained regions (HSR) containing no bands, regions of metaphasic chromosomes with abnormal bands, and small extrachromosomal bodies named double minutes (DM) (Baskin *et al.*, 1981). We note that MDR cells containing characteristic HSR and DM structures that contain amplified *mdr* genes lose the MDR phenotype if they are cultured in a medium that is devoid of drugs (Radziwill *et al.*, 1990). In the absence of a selective pressure, cells containing characteristic DMs rapidly lose both the DM character and

Table I. ATPase Activity of Reconstituted Human Pgp

Reference	Dong *et al.*, 1996	Sarkadi *et al.*, 1992	Ambudkar *et al.*, 1992
Type of human Pgp	CEM/VLB5[a]	mutant V185[b]	wild[c]
V_{max} (no drugs)[d]	0.2	1	5–12
Stimulation by verapamil (multiples of V_{max})	2	5	3

[a] Completely purified Pgp from highly resistant CEM cells.
[b] Partially purified Pgp overexpressed in SF9 insect cells.
[c] Partially purified wild type Pgp from plasma membranes.
[d] V_{max} is given in μmol ATP hydrolyzed per min and per mg Pgp (this value has been corrected for Pgp contents in the case of reconstitution into proteoliposomes).

drug resistance. On the contrary, MDR cells containing amplified genes that are integrated in HSR regions maintain their resistance level over long periods of time in the absence of drugs (Radziwill *et al.*, 1990). Consequently, both *mdr* gene amplification and the reversion of the MDR phenotype may carry potential clinical significance. It is important to note that to date no cytogenetic or molecular proof of gene amplification linked to the MDR phenotype in human tumors have been reported. However, hamster cells with low amplification levels of the *mdr* genes and high levels of specific mRNAs may lose their multidrug resistance when drugs are removed from the culture medium (Radziwill *et al.*, 1990). These cells may behave in the way human cells do in terms of MDR phenotype. *In situ* hybridization experiments of cDNA probes cloned from a resistant human tumor cell line to doxorubicin showed that sequences amplified 60 times were present

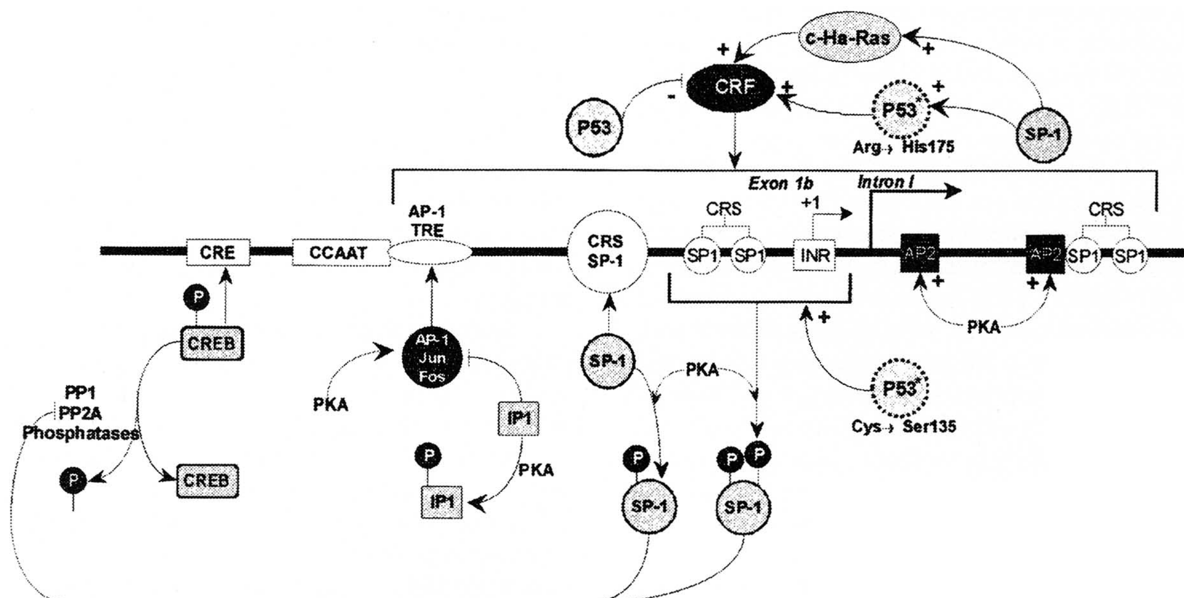

Fig. 2. The transcriptional regulation of the human *MDR*1 gene depends highly on protein kinase A (type I) activity (PKA). Following the PKA-dependent phosphorylation of regulation proteins that bind DNA such as the CRE-binding protein (CREB) on the cAMP regulatory element (CRE) or the Fos/Jun complex on the AP-1 site, the regulating sequences are *cis*-activated. The SP-1 protein, which normally activates the cAMP responsive sequence (CRS), may be phorphorylated by PKA. Hyperphosphorylation of SP-1 induces inhibition of phosphoprotein phosphatases (especially PP2A) that dephosphorylate CREB. This phenomenon yields an elevated transcriptional activity that is involved in the establishment of the MDR phenotype. If the tumor suppressor product p53 is wild type, the factors that respond to cAMP (CRF) are inhibited leading to a decreased transcriptional activity. On the contrary, when mutated p53 are present such as p53* (Arg175 → His) or p53* (Cys135 → Ser), CRFs are activated, which is followed by a stimulated transcriptional activity of *mdr*1.

in HSR regions characteristic of these cells (Fairchild *et al.,* 1987).

Control of the Human MDR1 Gene Expression. Comparison between Human and Murine Promoters

RNA analysis from human MDR cells showed that two distinct promoters control the transcription of *MDR*1 genes (Ueda *et al.,* 1987). The activity of the proximal promoter is located in about 1 kpb span on the 5′ side of the major transcription initiation sites (Ueda *et al.,* 1987), which are located between 130 and 140 bp upstream from the translation initiation site. Transcripts originating from the distal promoter have been found in cells selected with colchicine and also in several human tumors (Ueda *et al.,* 1987). These transcripts are rather rare for they contain sequences of the proximal promoter (included in exon 1A). In certain cell lines the activity of the distal promoter may represent 50–100% of the *mdr*1 transcription. The significance of the distal promoter is not yet known, even though it seems linked to drug treatment.

The proximal promoter region in human *MDR*1 genes contains a large variety of consensus sequences known to bind transcription factors such as a GC box, and a CAAT box about 200 bp upstream from the transcription initiation site. However, this promoter does not contain a TATA box even though a TFIID consensus sequence has been found 350 bp upstream from the initiation site. It has recently been shown that the human *MDR*1 promoter is a target for the oncogene product c-Ha-Ras-1 and for the wild tumor suppresser gene product p53, both of them being associated with tumor progression. c-Ha-Ras-1 induces a nonspecific stimulating effect on the promoter whereas p53 induces a specific repressor effect (Chin *et al.,* 1992). On the other hand, mutated p53 molecules induce a stimulating effect on the activity of this promoter (Zastawny *et al.,* 1993; Nguyen *et al.,* 1994) by acting from the 3′ region of the *MDR*1 downstream promoter (Strauss and Hass, 1995). Also, during tumor progression, mutation of the p53 gene has frequently been observed (Levine *et al.,* 1991). The wild type p53 gene codes a nuclear phosphoprotein that binds DNA (Kern *et al.,* 1991) and that plays an important role in negative regulation of cell growth and in tumor suppression (Finlay *et al.,* 1989). Since a binding consensus sequence has recently been defined for p53, this protein may be qualified as a transcription factor involved in the regulation of gene expression (Kunk *et al.,* 1992). It has been demonstrated that stimulation of signal transduction mediated by the cellular oncogene c-Raf-kinase increased the activity of the *MDR*1 promoter. This suggests that the endogenous *MDR*1 gene could be regulated by the c-Raf-kinase in response to proliferating signals (Cornwell and Smith, 1993). Moreover, the activation of the rodent *mdr* gene expression by v-*ras* and v-*raf* oncogene products has already been demonstrated for rat hepatocytes (Burt *et al.,* 1988). Negative regulation of the human *MDR*1 gene transcription has been reported upon specific binding of the NF-R1 protein to the two unrelated ATTCAGTCA and GC-box motifs (Ogura *et al.,* 1992).

Another interesting aspect of *mdr* gene regulation concerns the eventual role of apoptosis control on the MDR phenotype since modulation of apoptosis may influence chemotherapy and, thus, the outcome of cancer treatment. It has recently been shown that resistant ovarian cancer cells overexpress Bcl-2 and/or p53. Exogenous expression of Bcl-2 or a temperature-sensitive p53 mutant were shown to result in protection from drug-induced apoptosis and a delay in drug-mediated S-phase arrest of cell cycle. The corresponding *Bcl*-2 and *p*53 genes, together with *Bax* (the mRNA of which was found to be delayed in the presence of Bcl-2), may therefore modulate resistance to chemotherapy (Eliopoulos *et al.,* 1995).

Functional analysis of human *MDR*1 gene promoter is far from being complete and many questions still remain to be answered, such as, for instance, the tissue specificity of Pgp expression. It is not known either whether an interaction exists between the distal and the proximal promoters.

Studies concerning the proximal murine *mdr*1b promoter have shown that in the 5′ region of the promoter, a major transcription site was located 23 bp downstream of the TATA box (Cohen *et al.,* 1991). Several *cis*-acting elements have been discovered in the class I murine *mdr* genes (*mdr*1a and *mdr*1b). A TATA-like AT-rich sequence has been found in the promoter region of the two genes (Breathnach and Chambon, 1981), in contrast to the human promoter which is TATA-less (Ueda *et al.,* 1987). This important difference suggests that murine class I genes depend on the more precise regulation of transcription initiation than the human gene. Analysis of Chinese hamster TATA-less *Pgp*1 (hamster P-glycoprotein gene related to the MDR phenotype) transcription start point utilization revealed that drug-sensitive DC-3F cells utilize one major start point; in contrast, resistant sublines

were shown to "switch" to a more complex pattern using four additional transcription start points downstream from the major one (Ince and Scotto, 1995a). These observations suggest that the "switch" in transcription start point selection may be implicated in the increased expression of *Pgp*1 mRNA involved in the MDR phenotype. A new class of TATA-less RNA polymerase II promoters which share a similar arrangement of multiple start sites within a transcription "window" has recently been discovered (Ince and Scotto, 1995b). In all the promoters within this class, a DNA element called MED-1 is present downstream of the transcription window and is required for the activation of the multiple start sites (Ince and Scotto, 1996). The MED-1 consensus sequence found in human *MDR*1 promoter is just the reverse of that of the hamster *pgp*1 sequence, i.e., GGG AGC, and it controls the downstream multiple start sites, which represents a resistant cell specific feature found for the first time in the human *MDR*1 promoter.

A CAAT element has been identified in both class I murine *mdr* genes; *mdr*1a contains the pentanucleotide 5'CCAAT3' located at −78 pb from the transcription initiation site (Johnson and McKnight, 1989) and the *mdr*1b gene contains the canonical sequence 5'GGTCAAAT3' located at −80 bp (Benoist *et al.*, 1980). A GC-like box is recognized by the SP-1 transcription factor (Briggs *et al.*, 1986), whereas three of such boxes are present in the *mdr*1a gene.

Murine *mdr*1a and *mdr*1b and human *MDR*1 genes contain the consensus sequence AP-1 (Mitchell and Tjian, 1989) which is known to interact with the product of the proto-oncogene c-*jun* and its associated transactivator c-*fos*. It has been shown that c-*jun* expression was increased in precancerous lesions, in hyperplasic nodules, and also in hepatocellular carcinoma (Sakai *et al.*, 1989). Consequently, AP-1 elements may well induce a response to chemical stresses *via* the transcriptional activation of *mdr* genes since an AP-1 site has been discovered in the 5' region of the murine *mdr*1a and *mdr*1b genes and in the human *MDR*1 gene (Ueda *et al.*, 1987).

AP-2 elements have been identified in both murine *mdr*1a and *mdr*1b genes in different amounts and locations (Rickles *et al.*, 1989). An AP-2-regulated transcription involves activation of the protein kinase C (Roesler *et al.*, 1988), and, as has been shown, the protein kinase C-dependent phosphorylation of *trans*-acting factors is able to regulate the activity of the human *MDR*1 promoter (Uchiumi *et al.*, 1993).

A partially conserved AP-2 site in the murine *mdr*1b promoter is able to bind the 50-kDa human AP-2 protein isolated from HeLa cells (Mitchell *et al.*, 1989). However, the nuclear protein that binds this site in the murine *mdr*1b promoter is a 110-kDa molecule, thus appearing to be different from the AP-2 protein (Yu *et al.*, 1993). Neighboring regions surrounding the AP-2 site are GC-rich sequences and are preferentially recognized by the SP-1 protein (Briggs *et al.*, 1986). It has been observed that SP-1 expression is altered in human MDR leukemic cells selected with anticancer drugs (Borellini *et al.*, 1990), and SP-1 would not bind the region that contains AP-2 and GC-rich sequences (Yu *et al.*, 1993).

The human AP-1 protein binds the conserved AP-1 site in the murine *mdr*1b promoter. However, the effect of AP-1 on the *mdr* gene expression is yet unknown. Both this site and the previous one contain *cis*-acting regulatory elements.

The involvement of other transcription factors has recently been evidenced. The NF-Y transcription factor has been identified as binding to the Y-box sequence and proved to be a major factor in the regulation of murine *mdr*1b promoter. In parallel, the expression of CCAAT/enhancer binding protein b (C/EBPb) was shown to increase the level of *mdr*1b mRNA (Yu *et al.*, 1995). NF-Y appears to be an important factor for the *mdr*1b promoter, which may act in coordination with other factors such as C/EBPb for the control of the *mdr* gene expression. Moreover, a cell-type specific enhancer region spanning nucleotides −233 to −116 in the *mdr*1b locus has been shown to activate the *mdr*1b gene during hepatocarcinogenesis (Song *et al.*, 1995).

An activator element has recently been found within intron I of the murine *mdr*3 gene in P388/VCR-10 cells. This gene is transcriptionally activated after integration of a full-length murine mammary tumor virus within intron 1, upstream of the coding region of the gene. It was shown that this construct was capable of activating transcription from a cryptic site present in the antisense murine mammary tumor virus LTR (Lepage *et al.*, 1995).

A repetitive A/T sequence has been found in the *mdr*1b gene that is comparable to the repetitive LIMd of the 5' region in the *mdr*1a gene (Hsu *et al.*, 1990). Besides, important differences in the 5' region of both murine *mdr*1a and *mdr*1b genes have been noticed, which may explain their normal tissue-dependent differential expression (Arceci *et al.*, 1988). Both genes code for two isoforms of Pgp with distinct properties

(Lothstein *et al.*, 1989), suggesting that each isoform may have a unique function in different tissues. The induction of the *mdr*1b gene may be controlled by the glucocorticoid responsive element (GRE) that has been identified in the *mdr*1b gene since the progesterone receptor is able to bind the same sequence like the glucocorticoid receptor does (Strahle *et al.*, 1989).

WHERE A PECULIAR MEMBRANE LIPIDIC ENVIRONMENT MAY FAVOR THE MDR PHENOTYPE. POSSIBLE ROLE OF MEMBRANE CHOLESTEROL

The role and importance of cholesterol in multidrug resistance is a recent notion, even though it has been known for a long time that modification of membrane cholesterol concentration and of the regulation of cholesterol (and phospholipid) synthesis in tumor cells dramatically change the physicochemical properties of the affected membranes. Intracellular cholesterol homeostasis is underlain by a mechanism that is based on the antagonism of two classes of amphiphilic agents (Lange and Steck, 1994). Exogenous oxysterols (such as 25-hydroxycholesterol) may mimic excess of cholesterol by stimulating cholesterol esterification (Dashti, 1992), steroid conversion (Iida, *et al.*, 1989), and cholesterol secretion in the form of LDL from hepatocytes (Dashti, 1992), and reduce the activity of HMG-CoA reductase (Bell *et al.*, 1976). Like cholesterol, oxysterols inhibit the expression of genes that code for HMG-CoA reductase and for LDL receptors (Goldstein and Brown, 1990). A second class of components induce opposite effects: they inhibit cholesterol esterification, stimulate cholesterol synthesis, and counteract the action of oxysterols. These products are steroids of the progesterone class (Dashti, 1992), hydrophobic amines such as imipramine (Liscum and Dahl, 1992), and a large variety of amphiphilic agents most of which interact with Pgp: hydrophobic amines, phenothiazines, ionophores, colchicine, cytochalasines, and lysophosphatides (Lange and Steck, 1994).

Neosynthesized cholesterol is transported from the endoplasmic reticulum to plasma membranes according to a secretory-independent vesicular pathway that depends on energy (Urbani and Simoni, 1990). The reverse process has also been demonstrated (Lange, 1994). Agents such as progesterone, verapamil, and trifluoperazine highly decrease cholesterol movement from the plasma membrane to the endoplasmic reticulum (Lange, 1994; Nagy and Freeman, 1990) according to an unknown mechanism. This membrane cholesterol may well be the major substrate for the integral membrane enzyme of the endoplasmic reticulum acyl-CoA:cholesterol acyltransferase (ACAT) that catalyzes intracellular cholesterol esterification (Lange *et al.*, 1993). The inhibiting effects of Pgp inhibitors such as progesterone, verapamil, and trifluoperazine on the intracellular transport of cholesterol tested on CaCo-2 cells seem to suggest that Pgp is involved in the vesicular transport and the cellular traffic of cholesterol (Field *et al.*, 1995). This feature has recently been demonstrated by Metherall *et al.* (1996), who propose a model in which MDR P-glycoprotein is required for transport of sterols from the plasma membrane to the endoplasmic reticulum.

An increase in membrane cholesterol contents leads, among other biophysical changes, to membrane rigidification, which is at the origin of changes in transmembrane transport properties (Baggetto and Testa-Parussini, 1990). It has recently been shown that the decrease of membrane lipid fluidity due to the utilization of mild and nontoxic lipophilic surfactants such as Solutol HS-15, Tween 40, or Cremophor EL at doses of 10 μg/ml, induces the reversion of the MDR phenotype (Dudeja *et al.*, 1995). This suggests that these surfactants act directly on the binding of drugs on Pgp since partial Pgp solubilization is difficult to admit, as removal of the surfactant immediately restores resistance.

It has now been demonstrated that hydrophobic compounds interact with Pgp at the level of the lipid phase (Homolaya *et al.*, 1993). Lipophilic drugs such as adriamycin (a drug of the anthracycline family) enters the cell by passive diffusion through the lipid bilayer of cytoplasmic membranes (Ramu *et al.*, 1989). The development of resistance may thus be due, among other phenomena, to a modification of the concentration of membrane cholesterol which, in turn, impairs the membrane fluidity, thus interfering with the diffusion of drugs such as vincristine in the cytosol (Pallarés-Trujillo *et al.*, 1993). Altered membrane lipid compositions have been reported during the development of resistance to adriamycin in CHO cells (Rintoul and Center, 1984) and to vinblastine in human lymphoblastic cells (Rintoul and Center, 1984). More recently, it has been reported that the development of the MDR phenotype is associated with an altered cholesterol homeostasis (Mazzoni and Trave, 1993). When either sphingomyelin or cholesterol are added in equimolar quantities to phosphatidylcholine membranes, the transport of adriamycin decreases by respectively 60

or 80%. The inhibitory effect of these two lipidic compounds is probably due to a more compact membrane matrix. A more direct measurement has shown that cholesterol directly modulates the vincristine permeation through plasma membranes of MDR cancer cells (Pallarés-Trujillo *et al.,* 1993): the authors suggest that increased cholesterol/phospholipids ratio contribute to the decreased intracellular accumulation of drugs and to a higher resistance level of the MDR cells.

RESISTANCE AND ENERGY METABOLISM

Few studies have been made on this subject. It has been reported that human breast cancer cells *in vitro* with acquired doxorubicin resistance presented a 3-fold higher glycolysis than that of their parental cell line (Lyon *et al.,* 1988). In the same way, and very recently, the development of anthracycline resistance, and in particular doxorubicin, has been seen to be accompanied by enhanced glucose metabolism in Ehrlich ascites tumor cells (Miccadei *et al.,* 1996). This feature, evidenced by a higher rate of glucose phosphorylation and by increased activity of the pentose cycle, may be important in supplementing reducing equivalents necessary to detoxify against anthracycline aggression (Gessner *et al.,* 1990). In effect, it has been shown formerly that protection mechanisms against anthracycline cytotoxicity occur through both glutathione peroxidase and glutathione redox cycle (Kramer *et al.,* 1988, Lee *et al.,* 1988). The reducing equivalents used to regenerate reduced glutathione (GSH) come from NADPH, which is largely formed from by the pentose cycle. To corroborate this idea, Miccadei *et al.* (1996) found that in their anthracycline-resistant Ehrlich cells, the amount of glucose metabolized through the pentose cycle and the activity of both glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase were respectively 4.3 and 1.7 times higher than those found in sensitive Ehrlich cells. The same authors report an enhanced rate of both respiration and glycolysis inducing a 46% increased ATP production in anthracycline-resistant Ehrlich cells versus their sensitive counterpart. Because of the 54% lower doxorubicin contents in anthracycline-resistant Ehrlich cells, the enhanced energy requirement these cells exert may well be all devoted to doxorubicin extrusion through the ATP-dependent P-glycoprotein mechanism.

If we now examine the MDR phenotype from a bioenergetic point of view, it has been demonstrated that MDR transfectant cells are depolarized, i.e., they exhibit a lower plasma membrane electrical potential $\Delta\Psi$ and contain an alkaline intracytoplasmic pH (pH_i). Perturbation of these constants alter accumulation, partitioning, and retention of drugs. Thus it has been proposed that both a decreased $\Delta\Psi$ and an increased pH_i contribute to the altered cellular retention of chemotherapeutic drugs in MDR cancer cells (Roepe *et al.,* 1993). The same group recently showed that the rate of intracellular doxorubicin accumulation is related to $\Delta\Psi$ and that alkaline pH_i perturbations are important to determining the relative intracellular drug binding efficiency (Robinson and Roepe, 1996). (For reviews on this subject, see Roepe, 1995 and Roepe *et al.,* 1996.)

MODULATION OF THE TYPICAL MDR PHENOTYPE BY NEW THERAPIES

Pgp and the resulting MDR phenotype have been found to be inhibited by a variety of pharmacological compounds (Ford and Hait, 1993), suggesting the possibility for drug resistance modulation in human tumors. These inhibitors, called MDR modulators or chemosensitizers, belong to different classes such as calcium channel blockers (verapamil, tiapamil, nifedipine, prenylamine), neuroleptics (trifluoperazine, chlorpromazine, flupentixol), antidepressants (tricyclic, clomipramine), quinolines (chloroquine, quinine, quinidine), plant alkaloids (vindoline), steroids (progesterone), antiestrogens (tamoxifen), and surfactants (Tween-80, cremophor-EL) (Georges *et al.,* 1990). Cyclosporin A and its derivatives have been shown to bind Pgp (Foxwell *et al.,* 1989) and to restore intracellular drug retention in MDR cells (Boesch *et al.,* 1991). However, the drawback of the drugs used to inhibit the Pgp pump administered at doses necessary to be effective *in vivo* is that most of them become toxic, if they are not already intrinsically toxic such as the calcium channel blockers. This is due in fact, among other problems, to multifactorial resistance, which is most frequently encountered with MDR reversing agents. A safe and very efficient chemosensitizer has, therefore, yet to be discovered.

High-dose chemotherapy is representative of other approaches to overcome the MDR phenotype. This type of therapy, used to treat chronic myelogenous leukemia and malignant lymphomas, assumes that a dose–response relationship still exists at such high doses of the anticancer agent as to overcome resistance.

However, myelosuppression caused by chemotherapy is a major dose-limiting cytotoxicity in a large number of anticancer agents (Gale, 1988). Several techniques have been developed to decrease chemotherapy-induced myelosuppression, such as bone marrow transplantation (Vose and Armitage, 1992), liposome-entrapped drugs, antibody-directed drugs, radionuclides or immunotoxins, regional chemotherapy, chemoprotection, and hemopoietic growth factors (Gabrilove and Jakubowski, 1990). Reversion of the MDR phenotype has also been attempted in human colorectal adenocarcinoma adriamycin-resistant cells by long-term administration of a mixture of 18-mer antimessenger oligonucleotides targeted to adjacent binding sites of the *MDR*1 mRNA. These oligonucleotides were encapsulated into liposomes made of the synthetic cationic lipid DOTAP (Quattrone *et al.*, 1994). The amount of both *MDR*1 mRNA and Pgp was reduced by 50%. Resistant hematological malignancies can also benefit from the use of antisense oligonucleotides (Cucco *et al.*, 1996). When technical and delivery problems will be solved, this method may be an interesting way to clinically reverse the MDR phenotype. A promising approach to MDR circumvention has been attempted by using liposome-mediated transfer of hammerhead ribozymes aimed at the *MDR*1 mRNA, which significantly reversed the MDR phenotype of mesothelioma cell lines (Kiehntopf *et al.*, 1994).

Another approach to circumvent myelotoxicity due to chemotherapy has recently been proposed and consists of *MDR*1 gene transfer into bone marrow cells using retroviral vectors (Boesen *et al.*, 1994). Evidence that retroviral *MDR*1 gene transfer to normal hemopoietic stem cells would protect them from cytotoxic anticancer agents was first provided by *in vitro* experiments (DelaFlor-Weiss *et al.*, 1992). Many experiments have been conducted to show the effectiveness of *MDR*1 gene transfer. For instance, human $CD34^+$ cells have been transduced using amphotropic retroviral vectors containing the *MDR*1 gene, and the transgenic cDNA was detected in erythroid and myeloid clones derived from them (Ward *et al.*, 1994). After this gene transfer, increased resistance to high doses of taxol was shown in cell clones derived from the transduced $CD34^+$ cells (for a review see Koç *et al.*, 1996). Based on the findings of *in vitro* and *in vivo* animal studies, retroviral *MDR*1 gene transfer appeared to be safe and to deliver a good protection level of bone marrow from the toxic effects of intensive chemotherapy. After approval, the first clinical trials in the USA are now being performed on patients with advanced breast and ovarian cancer (Hesdorffer *et al.*, 1994, O'Shaughnessy *et al.*, 1994). Recently, to increase the safety of *MDR*1 gene transfer, a bicistronic vector containing thymidine kinase from herpes virus simplex (HSV-TK) as a passenger gene has been constructed (Sugimoto *et al.*, 1995). HSK-TK confers sensitivity to the antiviral agent ganciclovir, which also behaves like a suicide gene that will kill cells that inadvertently transduced.

Because of the properties of Pgp, an interesting application in molecular biology for efficient delivery and long-term expression of heterologous genes in animal cells has recently been proposed by S. Kane and coworkers: the authors describe a series of two-gene and bicistronic retroviral vectors using the human *MDR*1 gene as a selectable marker for the overexpression of a second heterologous gene of interest, such as a therapeutic gene, in transduced cells (Metz *et al.*, 1996).

In conclusion, because of their high rate of division, cancer cells are able to rapidly adapt their metabolism and their detoxification systems through genetic aberrations. In the case of resistant tumors, overexpression of Pgp may be regarded as an important tumor feature indicating that the marked cells have been conferred a selective advantage over other neoplastic cells because of their ability to decrease intracellular cytotoxic drug concentrations. This central anomaly is accompanied by other membrane, metabolic, and bioenergetic deviations to complete the MDR phenotype.

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