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MECHANISMS OF MULTIDRUG RESISTANCE IN CANCER TREATMENT

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Advanced breast cancer responds to a range of cytotoxic agents, but resistance always develops. Understanding the mechanisms of resistance may provide new therapeutic options. There are several major groups of resistance mechanisms. 1) The multidrug resistant phenotype. This is due to a membrane pump that can extrude a wide range of anticancer drugs - the P-glycoprotein. It is inhibited by a range of clinically used calcium channel blockers such as nifedipine and verapamil. Several other membrane proteins of 180 KD, 170 KD, 300 KD and 85 KD have been reported and are associated with MDR. 2) Glutathione transferances and detoxification mechanisms. These are a multigene family of enzymes that conjugate glutathione to chemically reactive groups. There are 3 major groups of enzymes—acidic, basic and neutral. They have been implicated in resistance to doxorubicin, melphalan cisplatinum chlorambucil and other alkylating agents. Other protecting systems include metallothionein and selenium dependent glutathione peroxidase. HSP27 confers doxorubicin resistance. 3) Topoisomerase II. DNA topoisomerases are involved in several aspects of DNA metabolism in particular genetic recombination, DNA transcription, chromosome segregation. They are a target for doxorubicin, mitoxantrone, VP16. Low levels of expression are associated with resistance. However, it is oestrogen inducible and this may be of therapeutic value. A novel topo IIb which is more drug resistant has been reported. 4) DNA repair. A score or more of genes are involved in the repair of DNA damage by drugs and radiation. Defective DNA repair may predispose to cancer of the breast and be responsible for adverse radiation reactions. Enhanced repair has been shown to be a mechanism of cisplatinum resistance. Several genes are inducible by DNA damage and may confer resistance e.g. A45. 5) Drug activation. Mitomycin C as well as cyclophosphamide and VP16 require activation for their effects. Low levels of cytochrome p450 reductase are associated with MMC resistance.

Key words: Drug resistance, topoisomerase II, cytochrome p450 reductase.

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The failure of chemotherapy to cure more than a minority of tumours is predominantly due to drug resistance. Resistance may be inherent or acquired; either as a stable change within the cell or induced following drug administration. Currently, although a majority of childhood tumours are curable, only a minority of adult tumours (e.g. Hodgkins and non-Hodgkins lymphoma, acute leukaemia, teratoma) respond fully. A significant prolongation of survival has been shown for small cell lung cancer, ovarian cancer and breast cancer, in latter case when chemotherapy is used as an adjuvant.

Multidrug resistance (MDR) is the phenomenon whereby exposure to one drug induces cross-resistance to a variety of agents to which the cell has not been exposed. Several mechanisms may mediate MDR, primarily expression of the MDR gene, topoisomerases and glutathione transferases.

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Most of the drugs used in breast chemotherapy have been implicated in multidrug resistance which may constitute a major factor in relapse. This review will concentrate on recent advances in the understanding of this phenomenon.

The hallmark of MDR is the expression of P-glycoprotein (PGP), a 170 kD protein with six hydrophobic domains and a tandemly duplicated ATP binding domain (1–4). PGP acts as a transmembrane exporter of drugs but the normal substrates have yet to be identified.

Homologies of PGP

Clear homologies exist between PGP and several bacterial transport proteins, in particular with conservation of the ATP binding domains (5). These proteins transport individual peptide and carbohydrate species into bacterial cells and are energised by hydrolysis of ATP. The transport protein Hly B exports α haemolysin protein from haemolytic bacteria. Its carboxy terminal contains the ATP binding site and is identical to P-glycoprotein in 50% of its amino acids.

In eukaryotes, one defined normal role for an MDR homologous gene has been found with the STE6 gene of Saccharomyces cerevisiae (6). The product of this locus actively transports the α factor mating pheromone (MAT). It does not confer a drug resistant phenotype. Another is a family of genes involved in antigen processing (7).

Chromosomal location and genomic organisation of MDR

In man the MDR1 gene is located on chromosome 7q 21–31 and contains an open reading frame of 1280 amino acids. There are two known human and three rodent MDR genes with control of expression of both human genes occurring at the levels of gene copy number, transcription, translation and post-translationally (1).

Molecular and genetic studies

The major evidence for PGP involvement in drug resistance has come through gene transfection studies. In man only the MDR1 gene has been shown to mediate MDR phenotype by transfection; MDR3 fails to confer any resistance. In rodents both PGP 1 and PGP2 may mediate resistance. There may also be involvement of an MDR homologue in mediating chloroquine resistance in Plasmodium falciparum (8).

Regulation of MDR

A variety of physical and chemical agents affect expression of MDR1, predominantly through increased transcription. The renal carcinoma cell line HDP 46 demonstrated an 8-fold increase in MDR1 RNA levels in response to heat shock, ethanol, sodium arsenite and cadmium, consistent with a role for PGP as a stress inducible gene product following environmental insults (9). These effects are not found in all cell types.

Furthermore, the differentiating agents dimethyl sulphoxide (DMSO) and sodium butyrate increased PGP expression in a variety of colon carcinoma cell lines as measured by Northern blots and immunoblotting (10). However, in one line (SW 620) increased expression was not accompanied by increased doxorubicin or vinblastine resistance. The regulation of MDR1 by gene amplification was suggested initially by the presence of double minutes and homogeneously staining regions (11). Several genes coamplify with MDR1 including sorcin, an acidic 22 kD protein homologous to calpain, which binds calcium, but no correlations have been found with resistance patterns.

Work in rodents has identified differential overexpression of MDR isotypes as a source of variable resistance patterns (12, 13). There are different RNA species detectable in resistant cell lines isolated after exposure to increasing concentrations of colchicine, vinblastine and taxol. The expression of different MDR genes encoding separate PGP isoforms may be a mechanism for generating diversity of response. Expression of these genes may occur through different pathways as MDR1a (MDR3) is primarily transcriptionally activated with MDR1b (MDR1) levels paralleling gene amplification. Furthermore, transfection experiments using mouse MDR1 and MDR3 confer overlapping but distinct drug specificities. In the former case preferential resistance to colchicine and doxorubicin is conferred, whilst the latter gave preferential resistance to actinomycin D.

Protein modification

Post-translational modification of MDR is also significant. In man a 140 kD precursor protein is gradually converted to a 170 kD form over 2 to 4 h. There are 10 potential N-glycosylation sites in PGP but only 3 appear on the external surface of the plasma membrane. Glycosylation deficient mutants of MDR cells show no change in resistance profiles compared with parental cells and the further finding that tunicamycin (a glycosylation inhibitor) does not affect resistance indicates that this may be of questionable significance. The PGP expressed in the mouse line J774-2 has been shown to be a substrate for cyclic AMP dependent protein kinase A with phosphorylation of serine and threonine residues having been demonstrated in vitro. PGP seems to be phosphorylated in its basal state by protein kinase C and this may be important in affecting drug transport. Drug accumulation assays of a multidrug resistant human carcinoma line showed that PMA treatment significantly reduced ^{3}H vinblastine accumulation induced by verapamil and that basal phosphorylation of PGP was increased 2-fold by phorbol ester treatment. However, it has also been demonstrated that staurosporine

and H7, which are inhibitors of protein kinase C and cAMP dependent protein kinase activity, do not affect overall PGP phosphorylation. Studies of vincristine resistant HL 60 cells demonstrated another membrane associated protein kinase (PK 1) which also phosphorylates PGP on serine and threonine residues and may regulate levels of multidrug resistance (14, 15).

Mutation of MDR1

Mutations within the MDR1 gene may alter patterns of cross-resistance. In KB cells a glycine to valine mutation at codon 185 resulted in a changed preferential resistance pattern from vinblastine to colchicine (16). This amino acid is located within the first hydrophobic region on the cytoplasmic side of the membrane and may be a part of a drug binding site. However, a recent study on KB lines transfected with both cDNAs also showed the 'mutant' PGP led to a significant increase in etoposide (VP16) resistance as well as increased resistance to vincristine, actinomycin D and taxol; reversal of resistance was achieved with 1 μM verapamil (17). Interestingly, studies using photoactive drug analogues showed that against expectations the mutant line binds less colchicine and more vinblastine than the wild type. This suggests that altered resistance may not be due to initial association of PGP with drug but by a subsequent dissociation of drug from PGP. This argues against an effect solely on the drug binding site and in favour of two critical points in PGP mediated translocation of drug: one in the drug binding site and another involved in intracellular drug localisation and transport.

RNA stabilisation

Recent evidence on the MDR1 gene in regenerating rat liver reveals another possible level of control with mRNA degradation being controlled by sequences in the non-coding region (18). Following hepatectomy a 2-fold increase in MDR transcription was demonstrated by nuclear run on experiments, whereas gene expression was increased 22-fold. Analysis of the 3′ encoding sequences of MDR reveals an AU rich sequence associated with unstable mRNA messages.

Summary of mechanisms of regulation

Expression of MDR is regulated by amplification, transcription and translation. The relative contributions of each may vary throughout the selection process. In a multidrug resistant ovarian carcinoma cell line overexpression of MDR1 mRNA was found without evidence of amplification at low levels of drug resistance (19). At intermediate drug concentrations, amplification became increasingly significant while at high drug level PGP increased without further changes in mRNA or gene copy number, consistent with translational modification or mutation. Apart from indicating that MDR may be mediated at different levels of protein in the same tumour under different selection pressures, this study also indicates that measurement of MDR1 mRNA alone may give a misleading impression of the amount of functional PGP present.

Tissue distribution of PGP

Studies on tissue distribution of PGP and MDR expression have involved immunohistochemistry with mouse and human monoclonal antibodies, ribonuclease protection assays and in situ mRNA hybridisation. Specific monoclonals have been developed to distinguish between the various PGP isoforms (20, 21). Nevertheless, problems of non-specific hybridisation continue to confuse results. The widely used C219 recognises highly conserved amino acid sequences found in all PGP isoforms characterised to date, including MDR 3; it also cross-hybridises to a small percentage of skeletal muscle fibres. Similarly, MRK 16 recognises external regions of the molecule but gives occasional staining of smooth muscle cells in the walls of the stomach and intestine. Recently it was found that treatment of cells with neuraminidase, thus affecting sialylation patterns, greatly increased the amount of detectable fluorescence by the antibody MRK 16 in samples of lymphocytes from CLL patients (22). Generally the highest levels of PGP have been found in kidney (lumina of the proximal tubules), adrenal cortex (zona fasciculata and reticulata), stomach, duodenum, colon and placenta. Expression occurs primarily in specialised epithelial cells with secretory or excretory functions on the luminal surfaces as well as in placental trophoblasts (23). Strong expression was also found in endothelial cells of capillary blood vessels at blood tissue barrier sites, primarily capillaries of the central nervous system and testes as well as the dermis (24). These observations have significance in light of the fact that the brain and testis constitute sanctuary sites in which relapse following systematic chemotherapy in conditions such as acute lymphoblastic leukaemia occurs, presumably because of failure of drug penetration. Location of PGP in these cells is primarily on the luminal surface consistent with the role of PGP in secretion of an, as yet, unknown substrate.

Clinical significance—expression in tumours

Studies of the significance of MDR in human tumours have recently become available. The most extensive study reported on levels of MDR1 mRNA in over 400 human cancers (25). Quantification of mRNA was measured using slot blot analysis comparing tumour samples to known drug sensitive (KB-3-1) and resistant (KB-8-5) cells. Expression was defined as common if MDR1 mRNA was detectable in over 50% of cancers in each group. The overall findings have been that there is an inverse correlation of levels of

MDR expression with chemosensitivity of the tumour type, though numerous exceptions have been found. Thus, for example, breast and ovarian cancers had lower levels of MDR and less frequent expression than colon and renal cell carcinomas. However, sarcomas and non small cell lung cancers had low levels of MDR and low frequency despite showing a resistant phenotype. This may reflect sensitivity of the assay and our ignorance of the level at which MDR expression becomes significant within the clinical context as well as the use of other resistance pathways. A study of 26 patients with myeloma, lymphoma and breast cancer showed highly significant correlations between positive PGP staining and in vitro resistance to doxorubicin (26). All 12 patients with PGP positive levels showed in vitro resistance as well as 3 out of 14 PGP negative subjects. No clinical data are available on these patients to determine in vivo significance.

Conclusion

To conclude, the majority of studies thus far have included too few patients to allow clinical significance of MDR expression to be fully clarified. Ongoing longitudinal studies will help to clarify as to whether identifying subgroups of tumour patients expressing MDR helps to predict response to chemotherapy and might therefore be appropriate for the use of MDR modulators. Problems are still significant with regard to correlative analysis of PGP and in vivo resistance profiles. Assays may be insufficiently sensitive to detect significant PGP expression; this may be particularly important in mRNA measurement where degradation of biopsy samples may occur. Similarly, as previously mentioned there have been difficulties in developing specific monoclonal probes.

Reversal of MDR

A variety of pharmacological agents reverse MDR and this has been extensively reviewed (27). These range from the calcium blockers, particularly verapamil and nifedipine to tamoxifen, phenothiazines and cyclosporin, among others. The concentrations of some drugs required to reverse MDR in vitro would produce toxicity in vivo and this has been a major limitation to effective clinical use of, for example, verapamil. Some of these agents probably act by binding to PGP and thereby cause intracellular accumulation of drug. Others, including phenothiazines, may act by binding calmodulin or do not affect efflux but cause redistribution within the cell. Normal basal to apical flux of vinblastine in multidrug resistant epithelial cells is reversed by verapamil with enhanced phosphorylation of PGP, but verapamil has also been noted to potentiate bleomycin, cisplatin and 5-fluorouracil toxicity which are not associated with the MDR phenotype. The most extensive studies on MDR reversal occurred with verapamil. For example, a study of calcium antagonists in multidrug resistant primary human renal cell carcinomas demonstrated significant enhancement of a vinblastine cytotoxicity with verapamil but high concentrations (1 μM) were required and this failed to abolish the tumour resistance completely (28). Recent studies on the use of verapamil in multiple myeloma have shown a significant improvement in remission rate and are encouraging (29). It is possible that using combinations of different MDR antagonists together at lower levels such as tamoxifen with verapamil may overcome MDR without the undue toxicity seen with the higher levels of each drug. Alternatively, chemotherapeutic drug analogues not binding to PGP, e.g. the doxorubicin derivative aclacinomycin, could be used. Further studies on whether higher doses of drugs can cure previously unresponsive tumours may be facilitated by studies of transgenic mice expressing high levels of PGP in bone marrow (30). Recently progesterone has been shown to bind to PGP in drug resistance human cell lines. Tamoxifen and toremifene have also been shown to reverse MDR and these group of drugs are attractive candidates for combination with other classes of MDR inhibitors (31–33). Although expression of P-glycoprotein thus far appears to be the most significant cause of the multidrug resistance phenotype, increasing attention has recently been paid to the so-called atypical multidrug resistance (at MDR). This has always been applied to circumstances where multidrug resistance phenotype has occurred without overexpression of the MDR gene. The commonest pattern appears to be of drugs which act on cellular topoisomerase II as a target.

Topoisomerase II

Topoisomerase II (topo II), the eukaryotic homologue of bacterial DNA gyrase, is a 170 kD homodimeric protein which plays a role in DNA replication, chromosome scaffold formation, chromosomal segregation, and possibly recombination and gene transcription (34, 35). In Saccharomyces cerevisiae, topo II null mutations are lethal because of the inability to separate chromosomes at mitosis. The enzyme acts by producing DNA single and double strand breaks and it attaches covalently to the 5′ ends of the break. Double strand breaks are staggered by 4 base pairs on opposite strands and the protein attaches as a dimer. Subsequently, strand passage occurs with changes in both supercoiling and DNA relaxation. In proliferating cells, the functional topo II is probably that which is incorporated into the salt insoluble nuclear matrix. In Drosophila topo II is enriched at the sites of attachment of chromatin loops to the nuclear matrix.

Topoisomerase II as a drug target

Several classes of drugs are termed topoisomerase inhibitors though they do not inhibit the initial stages of

topoisomerase action. These drugs include intercalating agents such as acridines (mAMSA), anthracylines (doxorubicin, daunomycin) anthracene-diones (mitoxantrone) and ellipticines. There is also an important group of non-intercalating agents which appear to bind directly to topo II, most notably teniposide (VP26) and etoposide (VP16). These drugs bind to topo II forming a cleavable complex; so-called because denaturation of the trapped protein with SDS or alkali reveals the DNA strand breaks with protein covalently linked to the 5-phosphoryl end of each broken strand. Since each complex requires two proteins, an increase in the amount of topoisomerase will correlate with increase strand breakage and potential toxicity. It has been shown, for example, that CHO cell lines hypersensitive to etoposide overexpressed topoisomerase II (36). Although the exact mode of cell death is unclear, presumably the cleavable complex prevents chromosome segregation as well as DNA synthesis. Almost all topoisomerase inhibiting drugs increase the number of topo II associated DNA strand breaks although this does not always correlate with cytotoxicity because the drugs may have other modes of action. Recent evidence suggests that the common mode of action of the structurally disparate anti topo II drugs is their inhibition of the enzyme's ability to religate its cleaved DNA intermediate (37). Thus, rather than forming the initial cleavable complex these drugs act by stabilising the ternary complex and prevent the final religation step.

Regulation of topo II

Topo II is expressed primarily during the proliferative phases of growth and is downregulated at plateau phase. The amount of topo II increases at the onset of DNA replication continues to increase through S and G2 phases, peaks in the late G2 to M phase and then drops after mitosis. At confluence topo II is switched off in untransformed but not in transformed cells. The regulation of topo II during the cell cycle may be relevant as to which phase is most sensitive to topo II inhibitors (38). Following serum stimulation of BALB-c 3T3 cells, actively proliferating cells showed much greater sensitivity to etoposide than quiescent cells. The increase in drug sensitivity began during S phase and reached a peak just before mitosis, with a maximal 2.5-fold increase in drug sensitivity. However, although the maximal number of topo II associated strand breaks occurred during G2, cytotoxicity was maximal during S phase, suggesting interactions with other mechanisms lead to cell death. Modulation of topo II activity during the cell cycle involves phosphorylation (39). This has been demonstrated for casein kinase and protein kinase C, and these changes may be crucial in switching on the activity of topoisomerase. Phosphorylation has been shown to lead to a 3-fold increase in enzyme activity and dephosphorylation inactivities the enzyme. Studies of ^{32}P incorporation into DNA topo II in vivo in chicken lymphoblastoid cells showed phosphorylation highest at G2 and M, suggesting phosphorylation may be involved in enzyme activation for sister chromatid disjunction. Glucose deprivation, hypoxia and the stress response to heavy metals have been shown to downregulate the amount of topo II while epidermal growth factor (EGF) is an upregulator (40). The latter effect may be simply due to causing increased cell proliferation.

Topoisomerase II and drug resistance

There has been demonstration of so-called atypical multidrug resistant phenotype in numerous cell lines. Characteristically, there is cross resistance to the full range of anti topo II drugs though not to the vinca alkaloids.

These mechanisms may include:

Point mutations and abnormally functioning topoisomerases. An HL60 human leukaemia cell line showed 100-fold resistance to mAMSA in contrast to 2- to 3-fold increase in etoposide resistance (41). Cleavage of DNA produced by mAMSA or etoposide had an absolute requirement for ATP in contrast to the situation in wild-type cells. A new restriction enzyme polymorphism was found in the resistant forms. A mutation at the ATP binding site has been found to induce mAMSA resistance in the bacteriophage T4 DNA topoisomerase (42, 43). The extent to which downregulation of the total amount of topo II is a significant resistance mechanism remains unclear, although reduced levels of topo II shown in some studies could be due to decreased half-life of the mutant protein. However, in a VP16 resistant mouse breast cancer cell line quantitative analysis of drug stimulated cleavage activity showed one-fifth of the wild-type topo II activity as measured by cleavable complex formation while mRNA slot blot analyses showed significant reductions within resistant cells (44). Several studies have demonstrated abnormal functional forms of topo II in resistant cells with different drug cleavage patterns (45). In a study of teniposide resistant human CEM lines, the amount of topo II bound to the nuclear matrix decreased due to a mutant enzyme which differs in salt stability, thereby affecting attachment to the nuclear matrix (46). It is probable that the functional topo II is that which is associated with the matrix, and with newly replicated DNA.

Methylation. Several studies have demonstrated hypermethylated sites. This could be of significance in regulating the transcription of non-mutant alleles (47).

Glucose deprivation and hypoxia. These factors may be relevant within the tumour mass and contribute towards decreased topoisomerase expression.

Cytosolic factors. A PGP negative human fibrosarcoma cell line was found to express 150-fold cross resistance compared to the wild type cells. The amount of topoisomerase II as measured by immunoactivity was only 3- to 10-fold lower in the resistant lines (DR4) (48). Nuclear

topoisomerase II function as measured by cleavage and decatenation assays was identical in both types. However, whole cell extracts of the resistant line showed significantly reduced VP16 induced DNA breaks implicating cellular mechanisms. A variety of antioxidant enzyme activities were increased in the resistent cells, including glucose-6-phosphate dehydrogenase, glutathione peroxidase and catalase. These enzymes may inactivate the free radical drug metabolites but their exact role in mediating resistance remains unclear.

Upregulation of topoisomerase I. This has been shown in several cases. Topoisomerase I, an enzyme which produces single strand breaks in DNA and is involved in transcription, may increase as a compensatory effect with topo II downregulation (as occurs in *S. cerevisiae*) and may be able to carry out the functions of topo II at least partially when it is downregulated.

Switch to a novel form of topo II (β). Purification of a topoisomerase II from mAMSA resistant P388 leukaemia cells indicated another form of topo II of 180 kD differing in several respects from the previously known 170 kD form. Partial cloning of the gene shows that it may lack the leukine zipper motif which is probably important in dimerisation of the 170 kD form and that it differs in expression throughout the cell cycle (topo II β levels peak during G1 and fall during the rest of the cell cycle), and in drug sensitivity (49, 50). The 170 kD topo II (topo IIα) is expressed in rapidly proliferating cells and its ratio increases compared to the 180 kD form (topo II β) in *ras* transfected NIH3T3 cells (51). As the 170 kD protein is preferentially sensitive to teniposide and merbarone, this could account for drug effectiveness against tumours which possess a higher percentage of topo IIα; i.e. transformed cells as opposed to normal cells. Similarly, switch of expression from topo IIα to topo IIβ could lead to an 8-fold degree of resistance with equivalent protein expression.

Tissue distribution of topoisomerase II

Studies of topo II activity in normal tissues showed the highest levels in spleen and thymus (52). Expression within tumours was highest in breast cancer and leiomyosarcoma. However, levels within these tumours were in the same range as normal tissues; possibly normal tissue topo II is relatively teniposide resistant. These results were surprising since one would expect the highest topo II levels to be in proliferating cells. Further studies on tissue levels are needed using antibodies and activity assays.

Clinical implications

Further studies are required to elucidate the clinical significance of altered topo II as well as possible methods of circumvention. It may be possible to reduce topo II resistance by molecular design. Mechanisms of resistance to the topo II inhibitor mAMSA include altered transport and MDR. By altering substituents on the anilino acridine nucleus resistance to both mechanisms was overcome (53). Upregulation of topo I could be significant as inhibitors, notably camptothecin, could be used and cells exhibiting this mechanism of resistance may be hypersensitive to topo I inhibitors. Nevertheless, evidence indicates that combined use of topo I and topo II inhibitors may, in fact, decrease cytotoxicity. This could be due to the inhibitory effects of camptothecin on cellular transcription. Another route could be through the development of competitive topoisomerase inhibitors rather than the current stoichiometric agents i.e. competition with topo II for binding to DNA would be more effective in low topo II expressers. There is possibly a link between MDR and topo II regulation with possible inverse correlation of these. A study of MDR induction showed simultaneous topo II reduction though this may simply be due to a generally inhibitory effect of the drugs on cellular metabolism rather than a specific action on topo II regulation (54).

Glutathione transferases (GST)

The involvement of glutathione and glutathione transferases (GST) in the multi-drug resistant phenotype has been difficult to clarify. Glutathione transferases are enzymes involved in detoxification, distributed in all organs but primarily in the liver and kidney. Their mode of action is by conjugation of glutathione (a tripeptide present in all organs in millimolar amounts) via the sulphur atom of its cystine residue to various electrophiles. GSTs may also act as intracellular binding proteins (e.g. for bilirubin and steroids) and may constitute up to 10% of the total cellular protein in the liver. In man, GST exists in cytosolic and microsomal forms. There are 3 major classes of cytosolic GST: π, α, μ. Several comprehensive recent reviews deal with classification of the GSTs and their involvement in drug resistance (55–58).

GST expression in drug resistance

Several anticancer drugs may be inactivated by GST catalysis e.g. melphalan, chlorambucil and cyclophosphamide. There have been numerous reports correlating increases in GST isozymes, particularly the π family, with the onset of drug resistance. There may be increased amounts of enzymes involved in glutathione synthesis and also glutathione peroxidase. GSTπ has also been thought to be significant as it constitutes the predominant isozyme found in human cancers with 2- to 4-fold increases in RNA levels in tumours of the colon, bladder, ovary and stomach relative to normal tissues, and it has been found to be overexpressed in several multi-drug resistant lines, particularly in doxorubicin resistant lines. Studies in ovarian cancer patients show expression of α and π isozymes following

treatment. However, studies have also shown little difference between GST in tumour tissue and normal cells. In a study of patients with CLL no correlation was found between chlorambucil resistance and GST expression which also did not vary significantly between control and CLL lymphocytes (59).

Transfection studies and gene amplification

Transfection of GSTπ cDNA into ras transformed NIH3T3 cells led to elevated GSTπ enzyme levels but although there was a moderate 1.8- to 3-fold increase in resistance to doxorubicin and ethacrynic acid (a substrate of GSTπ), there was no change in resistance to cis platinum, chlorambucil and melphalan (60). In another study, a doxorubicin resistant line of human breast cancer MCF7 with both GSTπ and MDR expression was analysed. The MDR and GSTπ cDNAs from these cells were cloned and transfected but although MDR conferred a resistance phenotype, GSTπ gave only moderate resistance to ethacrynic acid. The transfection of GSTπ, together with MDR, showed no significant additive effect. It may be that other enzymes of glutathione metabolism are important and glutathione peroxidase activity has been related to doxorubicin resistances (61). A chlorambucil resistant cell line was shown to have elevated basic transferases and cross resistance to melphalan and nitrogen mustard. The GST genes were amplified several fold and this provides evidence for a genetic change related to drug resistance (62).

Glutathione depletion

In view of the large amount of intracellular glutathione, it may be that non-enzymatic reaction of glutathione with electrophiles could be relevant. Several studies have shown that glutathione depletion with buthionine sulfoximine (BSO), a blocker of glutathione synthesis, potentiates melphalan toxicity in numerous cell lines. It has not been shown so far that GST activity changes amount to more than associated stress responses, following treatment with alkylating agents rather than definitive resistance mechanisms. However, the increased protection against doxorubicin, although small, has been consistently found to be correlated with GSTπ. It is of interest that transformation with vH-ras and v-raf oncogenes induces resistance to a number of cytotoxic agents and induces both PGP and GSTπ in NIH3T3 cells (63). Definitive evidence with regard to gene transfection experiments awaits cloning the GST isozymes into a variety of cell types and the testing of different drug classes. Evidence so far suggests that resistance induced would be less than 3-fold. However, coordinate changes reducing ability to regenerate or transport glutathione may interact synergistically with other resistance pathways. Studies are in progress involving the clinical use of BSO and ethacrynic acid which deplete glutathione. The role of other pathways in the mediation of multidrug resistance remains to be clarified. Although the platinum drugs and alkylating agents are not implicated with MDR expression, overexpression of metallothioneins occurs following chronic exposure to heavy metals and confers resistance to cisdiamminedichloroplatinum, melphalan and chlorambucil (64). Transfection of vectors expressing metallothionein conferred the same resistance profile.

Conclusion

Multiple mechanisms may be responsible for multidrug resistance. Furthermore, individual drugs may have several modes of action which could affect resistance. For example, doxorubicin is demonstrated to be located within multiple cellular organelles, suggesting several targets of action apart from the cell membrane. Even within the cell surface other mechanisms may be significant. An interesting recent study produced an doxorubicin resistant MCF-7 breast carcinoma line by exposing to drug in the presence of verapamil (65). PGP expression was not elevated but a novel uncharacterised 95 kD surface membrane protein was expressed. There was also a decrease in topo II activity which may have contributed to the resistance profile. Nevertheless, the presence of this protein in clinical samples of breast cancer refractory to ADR, suggests actions on membrane mechanisms other than PGP. Clarification of the various resistance pathways will enable the development of newer strategies for their circumvention as well as allowing more accurate predictive analyses of effective treatment regimens.

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