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Molecular alterations induced in drug-resistant cells

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The major obstacle to the ultimate success in cancer therapy is the ability of tumor cells to develop resistance to anticancer drugs. Several molecular mechanisms have been suggested to be involved in drug-resistance: a) decrease in the intracellular drug accumulation (increased activity of membrane transporters such as P-glycoprotein or multidrug-resistance-associated protein), b) changes in intracellular detoxification system (increased concentrations of glutathione or metallothioneins, or increased activity of related enzymes), c) alteration in nuclear enzymes (enhanced DNA repair and/or better tolerance of DNA damage, decreased activity of topoisomerases), d) altered expression of oncogenes (inducing increased level of protective molecules in cells or the inhibition of apoptosis). Drug resistance is a multifactorial phenomenon. The complexity of molecular alterations in drug-resistant cells is and will stay the main problem for the successful treatment of cancer.

Key words: drug resistance, tumor cells; cancer chemotherapy

Introduction

Chemotherapy is one of the proven strategies against malignant tumors, especially if the lesions are spread systematically. In many patients, first regimens are successful in reducing tumor size and are sometimes even able to eliminate all clinically detectable tumor masses. But most often, the successful treatments are relatively short lasting. In a vast majority, a certain number of tumor cells will survive and thus become a source of recurrent disease.

Chemotherapy of cancer may fail for various reasons. Among these, drug resistance is the most important one. This phenomenon was first observed by Sidney Farber (who introduced chemotherapy into cancer treatment) in 1948.[1] Almost fifty years later the molecular mechanisms involved in this process have been unravelled.

Resistance may be primary (intrinsic): the tumor cells do not respond from the start. Drug-resistance may be secondary (acquired): under the selection pressure of cytotoxic drugs tumor cells are able to develop certain mechanisms which render them resistant to these drugs. The tumor initially responds to therapy, but tumor growth resumes and the patient relapses.

Knowledge regarding the genetic nature and biochemical nature of drug resistance

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has been derived largely from cellular systems. By step-wise increase in the drug dose, highly resistant cell lines can be obtained. The mechanisms of drug resistance can be defined by comparing the biochemical and biological characteristics of parental and resistant cells.

A search for the cause or causes of drug resistance mechanisms has been occupying the attention of cancer researchers for more than four decades. Today it is known that several molecular mechanisms can be involved in drug-resistance. The most important one will be presented in this review.

Reduced intracellular accumulation: transport proteins

A broad spectrum resistance to cytotoxic drugs, termed multidrug resistance (MDR), involves simultaneous resistance to a wide array of natural, semisynthetic and synthetic compounds. The most common of them are shown in Table 1. They do not have similar structure or the same cytotoxic intracellular target, but are amphipathic and are preferentially soluble in lipid.

Multidrug resistance is caused by overexpression of a 170 kDa plasma membrane - associated glycoprotein (P-glycoprotein, Pgp; Table 1). It is an energy dependent efflux pump that decreases intracellular drug accumulation.[2-4] Pgps are coded by *MDR* gene family. The number of members varies between species. Human possess two *MDR* genes, *MDR1* and *MDR2*. Of these, only *MDR1* can confer a drug resistance phenotype.[5] Multidrug resistant cells, particularly those which display high levels of resistance, often possess an increased copy number of the *MDR1* gene.[2,4]

It was noted that Pgp bore a remarkable

Table 1. Characteristics of transport proteins: P-glycoprotein and MRP protein

Name	P-glycoprotein (Pgp)	MRP (multidrug resistance -associated protein)
Encoded by	*MDR1* gene	*MRP* gene
Mol. weight Length Number of amino acid	170 kDa 4.5 kb mRNA 1268	190 kDa 6.5 kb mRNA 1531
Discovered in	1970 year	1992 year
Resistance to	Anthracyclines, Vinca alkaloids, Podophyllotoxins, Colchicine, paclitaxel	Anthracyclines, Vinca alkaloids Arsenic and antimony-centered oxyanions
Normal tissues distribution (high levels)	Adrenal gland, kidney, liver, large intestine, pancreas, bile duct, lung, breast, prostate, gravid uterus	Testes, skeletal muscle, heart, kidney, lung
Tumor tissues distribution	Colonic, renal, hepatoma, adrenocortical, phaeochromocytoma	Leukemias (acute myeloid, chronic lymphocytic, acute myeloid, B-chronic lymphocytic),lung (NSCLC), anaplastic thyroid, neuroblastoma
Functions	Transport of xenobiotics Transport of hormones	Transport of leukotriene Transport of GSH-conjugates Transport of heavy metal oxyanions "MOAT" (multispecific organic anion transporter)

According to references [2-4] and [9]

structural similarity to bacterial transport proteins, particularly those transporting haemolysin.[6] Presumably, in evolutionary terms, it represents a highly conserved component of the cell. P-glycoprotein is detectable at a high concentration in certain normal tissues (Table 1). The disposition of the Pgp on the luminal surface of kidney brash border, on the mucosal surface of the large intestine, and on the bile canalicular surface of hepatocytes, indicates a normal role in transport and/or protection against exogenous toxins. High levels of Pgp were found in different tumors as well, specially in those arising from the normal tissue with a high Pgp level.[2-4]

Pgp overexpression has been associated with multidrug resistance in many drug-selected cell lines.[7] The final evidence that *MDR* gene is involved in multidrug resistance came from transfection studies: MDR1 gene inserted into retroviral expression vector confers a complete multidrug resistance phenotype.[5]

Recently, another member of the ATP-binding cassette transporter superfamily was isolated from non-Pgp small cell lung carcinoma cells, multidrug resistance -associated protein (MRP).[8] It also lowers intracellular drug accumulation, conferring a pattern of drug resistance similar to that of the resistance-conferring Pgps.[9] However, there may be some differences. For example, MRP confers only low resistance to paclitaxel and colchicine, which are reported among the best "substrates" for Pgp. Another notable difference is the ability of MRP to confer low resistance to arsenic and antimony-centered oxyanions. The characteristics of this protein are given in Table 1.

MRP has been identified in non-Pgp multidrug resistant cell lines from a variety of tumor types.[9] Transfection of an MRP expression vector into HeLa cells demonstrated conclusively that the protein conferred resistance to drugs.[10]

Some recent observations suggest that elevated MRP expression may occur prior to MDR.[11,12]

Decreased drug uptake

Decreased intracellular drug accumulation may occur due to decreased drug uptake, for drugs that enter the cells by the help of a cellular transport system. Loss or inactivation of this transport system may cause drug resistance, as it was observed for melphalan,[13] methotrexate[14] or cisplatin.[15]

Glutathione

Glutathione (GSH) is a simple tripeptide that contributes to more than 90% of intracellular non-protein sulphydryl compounds.[16,17] It is present in virtually all eucariotic cells. It is also synthesized by tumors, some of which exhibit high cellular levels of glutathione and high capacity for the synthesis of glutathione.

Glutathione plays an important role in cellular metabolism and in the protection of cells against free radicals induced oxidant injury (Table 2). It has been implicated in cell resistance to a number of cytotoxic drugs, particularly to alkylating agents and cisplatin.[18-22] There are a number of potential mechanisms by which GSH may affect cellular response to cytostatics. These include conjugation of electrophilic compounds, frequently catalyzed by the glutathione S-trasnferases (GST). In addition, GSH can detoxify oxygen-induced free radicals and organoperoxides using GSH-peroxidases.[23]

GSH may participate in the resistant phenotype in two ways. In cytoplasm it may bind electrophilic compounds, thus making them less dangerous. In nucleus, GSH may support the repair of the damage induced in DNA: by maintaining functional repair

Table 2. Functions of glutathione in metabolism and immune response

Functions	Antioxidant
	Conjugation with different compounds (exogenous, the products of metabolism)
	Amino acid transport
	Support of primary antibody response
	Regulation of T-lymphocyte proliferation
	Co-enzyme for multiple enzymatic reactions
	Thiol-disulfide exchange in protein synthesis and degradation
	DNA precursor synthesis
	Enzyme activation
	Regulation of microtubule formation
	Negative control of NF-kB activation
Deficiency	Increased sensitivity to irradiation and different toxic compounds
	Oxidant stress
	Cataract formation
	Impaired function of both T and B lymphocyte function and immune function in general

According to references [16] and [17]

enzymes or by maintaining deoxyribonucleotide triphosphate pool size.[24]

An argument for the potential role of GSH in resistance is based on the observation that the toxicity of many cytotoxic agents can be increased by lowering cellular GSH (as by adding specific inhibitor of GSH synthesis-buthionine sulfoximine).[25,26]

Glutathione S-transferases

Glutathione S-transferases (GST) are an important part of the Phase II detoxification system that metabolizes many lipophilic drugs and other foreign compounds, including anticancer drugs.[27-29] The overall result of this system is the conversion of lipophilic chemicals to more polar derivates, thus facilitating their inactivation and elimination. GST are abundant and together may constitute up to about 5 % of soluble cellular protein. Their importance supports the finding that they perform specific detoxification, structural and transport function in many phila: from bacteria to humans.

GST catalyze direct coupling of GSH to electrophilic drugs, thus making a less toxic and more readily excreted metabolic compound.

$$R\text{-}X + GSH \dashrightarrow R\text{-}SG + H\text{-}X$$

Figure 1. Conjugation reaction catalyzed by GSTs

Further, they may exhibit ligand binding function, by non-covalent binding of non-substrate hydrophobic ligands (such as heme, bilirubin, some steroids, and some lipophilic cytostatics.[27-29]

The diverse biological functions of GSH/GST system are mediated by multiple GST enzymes. The genome of most species encode several different isoenzymes of GSTs. In eucaryotic cells, there are five classes of GST. Four are found in cytosol, while the fifth class, the microsomal GST, is found primarily in the hepatic endoplasmic reticulum.

The microsomal GST are functional trimmers with molecular weights of 17 kDa. Cytosol GST are both mono- and heterodimeric complexes formed of GST subunits that range in size from 23 to 28 kDa. The classification of the cytosolic GST into alpha, pi and mu, and recently identified theta classes was originally based on physical and catalytic properties. Among them, GST pi was found at elevated levels in many tumor tissues relative to matched normal tissues.[30,31]

Several anticancer drugs have been definitively identified as GST substrates (Table 3). Therefore, it is not surprising that elevated levels of GST were found in cells resistant to some of these drugs like cisplatin, doxorubicin, melphalan etc.[27-29]

The final confirmation of GST involvement in drug resistance came from the transfection experiments.[32] The tranfection with

Table 3. Anticancer drugs: substrates for glutathione S-transferases

Direct evidence for involvement in drug resistance	Indirect evidence for involvement in drug resistance
Chlorambucil	Bleomycin
Melphalan	Hepsulfam
Nitrogen mustard	Mitomycin C
Phosphoramide mustard	Adriamycin
Acrolein	Cisplatin
BCNU	Carboplatin
Hydroxyalkenals	
Ethacrinic acid	
Steroids	

According to reference [27]

GST imparts a small but significant (and clinically relevant) increase in resistance to cisplatin (GST mu), doxorubicin (GST pi) or chlorambucil and melphalan (GST alpha).

Glutathione peroxidase

Glutathione peroxidase catalyzes the reduction of potentially toxic peroxides to alcohols by oxidizing GSH to its disulfide form (GSSG). GSSG is returned to GSH with the concomitant oxidation of coenzyme NADPH to $NADP^+$. GST enzyme can catalyze a selenium-independent GSH peroxidase activity leading to the detoxification of lipid and nucleic acid hydroperoxides. This redox cycle may play an essential role in protecting cells from damage of lipid peroxidation generated during normal metabolism or by redox recycling of many drugs.

Doxorubicin is one of the agents known to generate free radicals and peroxides. GST as well as the selenium -dependent enzyme GSH peroxidase can deactivate these metabolites through peroxidative mechanisms, resulting in decreased cytotoxicity. In tumor cells resistant to doxorubicin, increased levels of selen -dependent GSH peroxidase were found.[33,34]

Metallothionein

Metallothioneins (MT) were first discovered as a family of inducible proteins involved in $Zn2+$ and $Cu2+$ homeostasis and in the detoxification of heavy metals.[35,36] They are evolutionary conserved low molecular weight intracellular proteins with unusually high level of cystein content, that constitute the major fraction of the intracellular protein thiols.

Today is known that metallothioneins are a part of generalized cell response to environmental stress: the abundant nucleophilic thiol-rich groups in MT can react with many electrophilic toxins, participate in controlling the intracellular redox potential, and act as scavengers of oxygen radicals generated during the metabolism of xenobiotics. They can be induced by environmental stimuli such as epinephrine, glucocorticoids, thermal injury, cytokines, cyclic nucleotides, phorbol esters, UV light, etc., suggesting a protective function and a role in cell growth and proliferation.[35,36]

Metallothioneins are attractive candidates as modulators of cellular sensitivity to anticancer drugs. Elevated levels of MT have been observed in some malignant cells with acquired resistance to antineoplastic drugs, such as cisplatin.[37-40] Increases in intracellular MT by gene-transfer-produced resistance

to cisplatin, melphalan and chlorambucil, and less to doxorubicin and bleomycin[38] or N-methyl-N -nitro-N-nitrosoguanidine (MNNG) and methyl nitrosourea (MNU).[41] In most MT overexpressing cell lines, however, induction of MT did not cause a parallel increase in resistance. Therefore, the resistance associated with MT overexpression was not due to direct binding of the drug to MT. The study of Kaina and co-workers suggest that MT may participate as a cofactor or regulatory element in the repair or tolerance of toxic alkylating drugs.[41]

Nevertheless, increases in MT do not always result in a phenotype that is less sensitive to the toxic effects of antineoplastic drugs.[42] Thus, the protective role of MT has been questioned. Recently, transgenic mice lacking functional MT have been produced by homologous recombination of disrupted MTI and II genes. The embryonic cells of these mice exhibit enhanced sensitivity to cisplatin, melphalan, bleomycin, cytarabin or MNNG, confirming the protective function of metallothioneins against cytotoxic drugs.[43]

Gene amplification

One of the important mechanisms of drug resistance is gene amplification. The first observation of this phenomenon was noted by Biedler and Spengler. They found chromosome elongation in cultured hamster cells resistant to methotrexate (MTX) and called this extra DNA "homogeneously staining regions" (HSR).[44] Schimke and co-workers then showed that the extra DNA contains extra copies of the gene for the enzyme dihydrofolate reductase (DHFR), explaining the increased enzyme levels in the resistant cell.[45,46] The amplified DNA may either be present in chromosomes as HSRs or free, as minute chromatin particles usually present in metaphase spreads as pair minutes, called double minutes (DM).

After the initial demonstration that cells can overcome the MTX inhibition of DHFR by overproducing the enzyme by means of gene amplification, numerous other examples of this mechanism have been reported: for MDR,[2,4] for metallothioneins [35,36] etc.

DNA repair

The resistance to some cytotoxic drugs can be caused by enhanced ability of cells to repair the DNA induced damage or to tolerate their presence. One of the most studied phenomena in this respect is resistance to cisplatin.

It has been well established that cisplatin binds to DNA and that these adducts contribute to cellular toxicity. In a number of cisplatin resistant cell lines, an enhanced repair of DNA lesions has been demonstrated.[21,22] Thus, Eastman and Schulte provided direct evidence for increased repair showing that the predominant lesions, cis-GG adducts, were more rapidly removed from resistant than sensitive cells.[47] These resistant L1210 cells can also reactivate a cisplatin-damage plasmid more readily than sensitive parental cells.[48] However, a correlation of repair activity with drug-resistance has not always been demonstrated: L1210 cells with 100-fold resistance to cisplatin, removed cis-GG intrastrand adducts only slightly better than 20-fold resistant cells.[47]

Repair of platinum damage in very specific regions of the genome is a possible characteristic of enhanced repair in resistant cells. If only active genes are more efficiently repaired in resistant cells, then it is not likely that a significant change in overall platination levels or repair rates will occur. Enhanced gene-specific repair could explain some of the controversial results found in such investigations. While preferential repair of the interstrand cross-link in active versus

inactive regions was not found in Chinese hamster ovary cells,[49] it was demonstrated in resistant human ovarian 2008 cells.[50]

It was observed that some cisplatin-resistant cell may have higher DNA platination than parental cells,[51] or may tolerate several fold more platinum on their DNA at equitoxic concentrations as sensitive cells.[52,53] Considering these facts, the concept of enhanced tolerance to DNA damage was suggested as a potential mechanism of resistance. However, the basis of this phenomenon is not well understood.

Several groups have described DNA-binding proteins that retard the mobility of cisplatin-damaged DNA fragments in non-denaturing polyacrylamide gels. It has been hypothesized that these proteins are either involved in DNA repair by shielding adducts from repair, or are involved in transcription.[54] A number of cisplatin-resistant cells have been investigated for changes in these DNA damage-recognition proteins.[22] However, no obvious correlation between the expression of DNA damage-recognition proteins and resistance to cisplatin was found.

Some of the recent papers suggest that resistance to DNA damage can be acquired via the loss of DNA mismatched repair activity. The DNA mismatch repair system acts after DNA replication and corrects non-Watson-Crick base pair and other replication errors. Human cells lacking mismatch repair activity have high spontaneous mutation rates. Also, they may be resistant to certain cytostatics, such as etoposide,[55] cisplatin[56] or N-methyl-N-nitro-N-nitrosoguanidine.[57]

DNA toposiomerase

Besides MDR, some resistant cell lines exhibit atypical multidrug resistance (at-MDR). At-MDR is distinguished from the MDR in the following ways: a) lack of cross-resistance to the *Vinca* alkaloids,[58] b) absence of a drug accumulation defect,[58] c) relative insensitivity to modulation of resistance by verapamil or chloroquine typical inhibitors of P-glycoprotein,[59] and d) lack of overexpression of the MDR1 gene or its product, Pgp.[59]

At-MDR involves altered activity of topoisomerases II. Topoisomerases II are enzymes that catalyze changes in the secondary and tertiary structures of DNA. They are necessary for replication, recombination and transcription, as well as in mitotic chromosome condensation and segregation. Topoisomerases II act via introduction of a transient double-stranded break in one segment of a DNA molecule through which a second DNA duplex is passed before religation of the break.[60]

The levels of these enzymes are markedly higher in exponentially growing than in quiescent cell lines. Two distinct forms of topoisomerase II exist in human cells, termed α (170 kDa form) and β (180 kDa form).[61] They differ not only in molecular weight but also in their patterns of expression and their apparent sensitivity to anticancer drugs.[62] In cell lines the expression of the α isoform has been shown to be strictly proliferation dependent, whereas the β isoform is presented in both dividing and non-dividing cells.

There are some inhibitors of topoisomerase II (doxorubicin, epirubicin, mitoxantrone, etoposide, teniposide) that trap the "cleavable complex" resulting in increased DNA scissions and inhibition of rejoining.[60,63,64] These protein-associated DNA lesions are directly toxic to cells. The cells with a high level of topoisomerase II are generally more sensitive to inhibitors than cells with a low level of these enzymes.

There is a number of rodent and human tumor cells lines in which resistance to topoisomerase II inhibitors are connected with decreased level of the topoisomerase II α and /or β. The resistance mechanisms appear to be the result of a decrease in the activity of topoisomerase II.[63-68]

Beside topoisomerase II, drug resistance may involve the altered activity of topoisomerase I. Topoisomerase I is an enzyme abundant in actively transcribing gene regions. It has important role in DNA replication and elongation step of transcription. Contrary to topoisomerase II, toposiomerase I introduce a transient single-stranded nick in DNA and is ATP independent. Several cytostatics, such as camptothecins and actinomycin D are the poisons of topoisomerase I.[60,63,64] Drug induced accumulation of topoisomerase I-DNA cleavable complex is directly proportional to drug cytotoxicity and antitumor activity. Resistance to topoisomerase I inhibitors involves altered activity of this enzyme, that may be caused by mutation(s) in the gene coding for topoisomerase I.[69]

Oncogenes and tumor suppresser genes: signal transduction pathway and apoptosis

In last few years interest has been focused on oncogenes and their role in drug-resistance. The direct evidence that oncogenes can be involved in drug-resistance came from transfection studies. The transfection of murine NIH3T3 cells [70,71] or kerytocytes[72] with *ras* oncogene resulted in resistance to cisplatin. *Ras* oncogene may induce resistance to doxorubicin as well.[73] Moreover it was found that the degree of cisplatin resistance correlated directly with the level of c-*myc* expression,[74,75] while the re-establishment of the normal level of c-*myc* transcription restored original sensitivity.[74] C-*myc* oncogene was also involved in resistance to methotrexate.[76] Using ribosome mediated cleavage of c-*fos* mRNA, the role of c- *fos* oncogene in resistance to cisplatin was proved.[77] Resistance to cisplatin was achieved by the transfection of *src* oncogene as well.[78]

The mechanisms by which oncogenes cause drug-resistance in not quite clear.[79] It has been suggested that c-*myc* oncogene binds to DNA, and thus directly or indirectly regulates a process of DNA repair.[74] *Ras* oncogene might induce resistance by regulating the expression of other genes involved in the protection of cell against cytostatics. It was shown for glutathione transferase pi,[80] topopisomerase II,[81] c-*jun*,[82] glutathione,[83] MDR,[84] or altered membrane potential.[85] Scanlon hypothesized that *fos* expression is the trigger that causes the resistance response (primary DNA reparability, as indicated by DNA polymerase α, DNA polymerase β, thymidilate synthase, DHFR and topoiosmerase I expression). Consistent with this concept is the observation that transfection of sensitive cells with c-*fos* generated eithold resistance to cisplatin,[77] while attenuation of the elevated c-*fos* expression returned the cisplatin toxicity to that of parent population. Another oncogene, mutated *p53,* may confer resistance to many hydrophobic drugs by stimulating specifically MDR1 promoter.[84]

It must be mentioned, however, that not always an increased expression of *ras*, *myc* or other oncogenes caused an increased resistance to cytostatics.[75,86-88]

In many cases the cellular damage caused by active doses of drug is not sufficient to explain the observed toxicity. Therefore, it is possible that some determinants of inherent drug sensitivity and resistance may be independent of those which involve the formation of the drug-target complex and its immediate biochemical sequel, such as commitment to cell death. Cell death is activated by natural control processes whose function is to allow repair of low level damage to DNA while eliminating those cells in which repair is not possible. There are two modes of cell death: apoptosis and necrosis. They differ morphologically and biochemically. Necrosis is associated with cell swelling, rupture of membranes and dissolution of organized structure. That is a consequence of the loss of osmoregulation. DNA degradation occurs at

a late stage. In contrast, in apoptosis internucleosomal cleavage of genomic DNA and chromatin condensation precedes the loss of membrane integrity (Figure 2). Necrosis mostly results from a major cell insult such as that caused by serious mechanical, ischemic, or toxic damage. Apoptosis generally occurs as a response to less severe injury and is also involved in the development and remodeling of normal tissue.[89, 90]

Apoptosis induces a wide variety of cell stresses and cytotoxic chemicals,[89,90] among them anticancer drugs.[91-94] Deregulation of normally integrated cell cycle progression appears a central signalling event in most forms of apoptosis.[90]

Apoptosis is a highly conserved active mechanism that requires the expression of several specific genes. Also their exact function is not quite understood, certain genes have been proposed as positive (*p53*, c-*fos*, c-*myc*, interleukin-1 converting enzyme etc.) or negative regulatory elements (*bcl-2* or glutathione redox cycle). They induce or prevent the onset of apoptosis.[95-103] Among them, *p53* and *bcl-2* are the most important and most studied.

p53 protein can function as a genetic switch capable of activating G_1 arrest, resulting in the repair of DNA damage.[94] Also, it is required for efficient activation of apoptosis following irradiation or treatment with chemicals. Loss of p53 function has been reported to increase resistance of tumor cells to a variety of cytotoxic drugs.[95,96] Recently it was shown that cells with mutated *p53* gene display perturbed G_1 arrest or apoptosis. This defect appears to reduce the sensitivity to DNA-damaging agents, suggesting that inhibition of apoptosis may represents a mechanism by which tumor cells may acquire drug-resistance.[95,96] By transfer of normal p53 into p53-defective non-small cell cancer line, an important increase in sensitivity to cisplatin was determined, which was related to the promotion of apoptosis.[97] However, the paper recently published by Wosikowski *et al*. suggests that alterations in p53 gene status or protein functions are not critical for the development of multidrug resistance.[98]

On the other hand, Bcl-2 protein inhibits apoptosis [99,100] and increases cell resistance to drugs.[101] Recently, *bcl-2* related gene products have been reported. One of them, Bax, homodimerizes as well as heterodimerizes with Bcl-2 protein. The Bcl-2:Bax ratio may determine survival or death after an apoptotic stimulus.[102]

Therefore, oncogenes and tumor supressor genes may be involved in drug resistance in two ways: by increasing the level of protective molecules in cells or by inhibiting apoptosis.

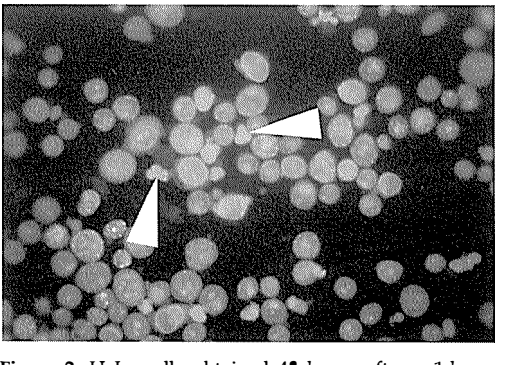

Figure 2. HeLa cells obtained 48 hours after a 1-hour treatment with 150 μM cisplatin. Apoptotic cells showed typical chromatin condensation, fragmented nuclei and cellular shrinkage (arrowhead), while intact nuclei exhibit "mottled" fluorescence. From reference [91].

Other factors

In doxorubicin treated cells an altered pattern of intracellular drug distribution was observed. The initial accumulation of drug in perinuclear location was followed by the development of a punctate pattern of the drug scattered throughout the cytoplasm. This pattern was suggestive of a process of drug sequestration, possibly followed by vesicle transport. In resistant cells, alteration in

the intracellular drug distribution was accompanied by a decrease in nuclear *versus* cytoplasm drug ratio.[104,105]

There is more and more evidence, that drug resistance is a multifactorial phenomenon (only for very low doses of the drug a single mechanism can be involved in drug-resistance. Thus, for instance, in cells resistant to cisplatin altered drug accumulation, increased levels of glutathione and related enzymes, metallothioneins, increased repair and altered expression of oncogenes could be observed.[21,22,39,40] In methotrexate resistant cells, decreased uptake of this drug, decreased polyglutamation, decreased affinity to DHFR and increased levels of target enzyme are the most common cause of drug resistance to MTX.[106] It should be mentioned, however, that all of these mechanisms need not be induced in drug resistant cells. So, in cisplatin resistant human laryngeal carcinoma cells only decreased platinum accumulation was connected with resistance to cisplatin, while no alteration in oncogene expression, no involvement of glutathione, glutathione transferase or metallothioneins was determined.[40,87]

Due to induction of several protective molecular mechanisms, resistant cells obtained after treatment with a single drug, can become resistant to various unrelated drugs (Table 4). The schedule of drug-resistance development can also influence the resistance pattern. Even with the same treatment schedule, clones with different cross-resistance patterns occur.[109]

It is generally accepted that the resistance to drugs can be induced by treatment with chemicals. However, in last several years it became obvious that also ionizing irradiation can induce drug resistance in irradiated cells [110-114] by the same mechanisms that are involved in resistance development induced by cytostatics.[112,114-120] This fact, if supported *in vivo*, and specially in clinic, is of the outmost importance for the patients. Namely, if irradiation precedes chemotherapy, it can reduce the success of combined therapy.

In conclusion, drug resistance is a complex, multifactorial phenomenon, which may involve decreased intracellular drug accumulation, increased detoxification, increased DNA repair, decreased activity of toposiomerases, gene amplification, altered onco-

Table 4. Drug sensitivity pattern of resistant cell lines

Cell line	Drug used for resistance development	Treatment schedule	Resistant to	Sensitive to
Laryngeal carcinoma[1]	vincristine	acute	DOX, MTX	CDDP
		continuous	DOX, MTX, 5-FU	CDDP
Cervical carcinoma[2]	cisplatin	acute	VCR, DOX, ETO, MTX, 5-FU	
		continuous	VCR, MTX	
Laryngeal carcinoma[3]	cisplatin	acute	VCR, MMC	ETO, DOX
		continuous	VCR, MMC, 5-FU	
Breast adeno-carcinoma[4]		doxorubicin	continuous	VCR, VBL, CDDP, CBDCA, (MMC, 5-FU)*

[1] reference 107, [2] reference 39, [3] reference 108, [4] reference 88.
* Significant resistance only at higher doses.
Acute = 1 hour treatment; continuous = 24 hours treatment
VCR= vincristine, VBL = vinblastine, DOX = doxorubicin, ETO = etoposide, MTX = methotrexate, 5-FU = 5-fluorouracil, CDDP= cisplatin, CBDCA= carboplatin, MMC= mitomycin C.

gene and tumor supressor gene expression, as well as inhibition of apoptosis. Resistance pattern of anticancer drugs is determined by the a) genotype of the cells, b) genotoxic agent involved in resistance development, and c) treatment schedule. The complexity of drug-resistance mechanisms, as well as sometimes conflicting experimental data suggest the need to continue such investigation and clarify the cascade of events involved in this process.

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