Drug Resistance in Lung Cancer

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Summary

Drug resistance is an important problem in the treatment of patients with cancer. Tumors become resistant not only to the drugs used initially, but also to those to which they have not yet been exposed. Data obtained from various sources indicate that multiple mechanisms contribute to drug resistance. Many of them are mutually related to each other. Resistance-related proteins such as P-glycoprotein, multidrug resistance related protein, lung resistance related protein, glutathione-dependent enzymes, topoisomerases, metallothioneins, thymidylate synthase and O6-alkylguanine-DNA alkyltransferase have been found in different human lung tumors, but these alone cannot explain the drug-resistant phenotype. Cell-cycle-related proteins, angiogenic factors, protooncogenes, and tumor suppressor genes can also contribute to the manifestation of drug resistance phenotypes. In future, a key challenge will be to determine the relative quantitative contributions of each of these mechanisms to overall resistance. The use of DNA microarray technology will yield insight into the mechanisms of drug resistance and facilitate the rational design of more effective strategies to circumvent resistance.

Key words: Drug resistance, lung cancer, resistance-related proteins, protooncogenes, DNA microarray

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I. Introduction

Lung cancer is a global problem because it is the most frequent cancer in the world, and the incidence of lung cancer is still increasing (0.5% per year). Consequently, lung cancer will remain a major cause of world cancer death in the 21th century (Haugen, 2000). The prognosis of lung cancer is very poor: nearly 80% of patients die within 1 year of diagnosis. Despite major advances in patient management, chemotherapy and radiotherapy made over the past decades, long-term survival is reached only in 5-10% of the patients (Richardson and Johnson, 1993; Mattern et al, 2002). The major problem in lung cancer chemotherapy is the manifestation of inherent and acquired drug resistance of the cancer cells. Resistance to anticancer agents concerns small cell lung cancer (SCLC) as well as non-small cell lung cancer (NSCLC). Most patients with SCLC have an initial response to chemotherapy, but the majority relapses and their tumors is largely refractory to further treatment. NSCLCs are usually inherently resistant and are generally nonresponsive to initial chemotherapy.

II. Definition of drug resistance in vivo and in vitro

Clinical drug resistance criteria are defined on the basis of tumor response criteria documented by the World Health Organization (WHO). When a patient does not show more than a partial response after chemotherapy treatment, the tumor is considered to be resistant to the anticancer drugs used. The criteria for in vitro drug resistance are not clear, in most studies, the 50% inhibitory concentration (IC50) values are established. Different in vitro test procedures have been used to test the sensitivity or resistance of lung tumor cells to cytotoxic drugs prior to therapy. The soft agar clonogenic assay (Hamburger and Salmon, 1977), the most widely used test system for in vitro drug selection, has proven to be of limited practical use in lung cancer since drug testing can be performed in only a minority of cases due to technical problems. Renewed interest in in vitro testing has been generated by the use of dye exclusion assays which are simple colorimetric tests of cell proliferation and survival (Weisenthal et al, 1983). The use of xenografts for testing drugs in vivo has also been recommended (Mattern et al, 1984; 1987; 1988). In most instances good agreement was found between the in vitro and the in vivo results. It is common to all test systems used that tumors which demonstrate resistance during testing will not, with a high degree of probability, respond to therapy in the clinic (Mattern et al, 1982). Thus, lung cancers exhibit a drug resistance profile in vitro paralleling that observed in clinical practice. Cell lines established from untreated SCLC are often sensitive in vitro to cytotoxic drugs whereas cell lines from NSCLC (sqamous cell carcinomas and adenocarcinomas) or relapsed SCLC are resistant to multiple agents (Carmichael et al, 1985; Ruckdeschel at al., 1987). In an effort to better understand the mechanisms of drug resistance, many in vitro selected lines have been generated by continuous or pulsed exposure to drugs. Such induced drug resistance has been associated very often with changes in expression of certain intracellular and plasma proteins which are partly characteristic for resistance to certain drugs.

III. Specific mechanisms of drug resistance.

A. Multidrug resistance

One mechanism of resistance which has now been well characterized is the so-called multidrug resistance (MDR) which describes the broad pattern of crossresistance to drugs. Two types of multidrug resistance have been described: First mediated by P-glycoprotein (so called Pgp multidrug resistance) and second which is associated with all other mechanisms apart from Pgp (non Pgp multidrug resistance). These mechanisms include expression of: other ATP dependent transporters such as multidrug resistance related protein (MRP), lung resistance related protein. Expression of proapoptotic and antiapoptotic proteins together with DNA repair mechanisms and drug detoxifying systems also play a part in non Pgp mediated MDR.

Pgp multidrug resistance

P-glycoprotein is a 170 kDa membrane-associated glycoprotein (P-glycoprotein) encoded by the *mdr1* gene (Gros et al, 1986). Based on the homology of the *mdr1* gene to a variety of genes encoding membrane transport proteins and on the ability of P-glycoprotein (P-gp) to bind drugs, it is believed that the multidrug resistance results from the function of P-gp as an energy-dependent drug efflux pump (Bradley et al, 1988). Relatively high levels of P-gp/mdr1 expression have been shown in many intrinsically drug-resistant tumors derived from tissues which normally express P-gp, including adrenal gland, kidney, liver, colon and rectum (Fojo et al, 1987). Generally low, but detectable levels of *mdr1* gene expression on performance of RNA slot blot analysis or RT-PCR have been found in sqamous cell lung carcinomas as well as in normal lung tissue despite the intrinsic chemoresistance of lung cancer (Lai et al, 1989; Abe et al,1994). Other studies using similar techniques also demonstrated that lung tumors contain low or undetectable levels of mdr1 mRNA (Goldstein et al, 1989; Shin et al, 1992). In contrast, using various monoclonal antibodies reactive with P-gp, some studies have revealed a relatively high expression of this protein in lung cancer (Radosevich et al, 1989). The inconsistency with the very low mdr1 mRNA levels found in lung tumors may be attributed to the varying sensitivity of the different methods to detect mdr1/P-gp expression (Herzog et al, 1992). Several SCLC and NSCLC cell lines made resistant to some MDR related drugs or derived from tumors relapsing after chemotherapy have been shown to express mdr1 mRNA and P-gp (Lai et al, 1989; Twentyman et al, 1986). In NSCLC, there appears to be a relationship between P-gp expression and drug resistance in vitro (Volm et al, 1991). In our studies we did not find a significant relationship between any histological subtype of NSCLC and preferential expression of Pgp and other MDR related proteins such as MRP-1 (multidrug resistance protein), Table 2. The significance of mdr1/P-gp expression as a predictor for response to therapy in lung cancer is not yet elucidated. Studies including larger numbers of patients are necessary for solution of this problem.

In conclusion, there is no evidence to suggest a major role of P-gp in the MDR of lung tumors. The overexpression of mdr1/P-gp alone does not completely explain intrinsic MDR, and thus other drug resistance mechanisms are thought to exist in lung cancers.

Table 1: List of multidrug resistance- related proteins and anti cancer drugs which they can influence.

protein	amplification/overexpression	Resistance to
P-glycoprotein	+	DOX, ACT, VCR, VP16, VM26, COL, VDS, VBL,MTC
Topoisomerase II	-	DOX, ACT, VP16, VM26, MITOX, m-AMSA
Glutatione-S transferase-π	+	DOX, ACT, VP16, VM26, L-PAM, DDP, CTX, CHL, BCMU
Metallthionein	+	DOX, DDP, L-PAM, CHL
Thymidilate synthase	+	5-FU, MTX, DOX, DDP

Abbreviations for explanation of the generic names of the above mentioned drugs : ACT, actinomycin D; ARA-C, cytosine arabinoside; m-AMSA, amsacrine, BCNU, carmustine; CCNU, lomustine; CHL, chlorambucil; COL, colchicine; CTX, cyclophosphamide; DDP, cisplatin; DOX, doxorubicin; 5-FU, 5-fluorouracil; HU, hydroxyurea;
MITOX, mitoxantrone; MTC, mitomycin C; MTX, methotrexate; L-PAM, melphalan; PC, procarbacin; VBL, vinblastine; VCR, vincristine; VDS, vindesine; VM26, tenoposide; VP16, etoposide

Table 2. Expression of multidrug resistance related proteins within individual stages and histological subtypes of NSCLC .
(Škarda J, Hajduch M 2006 unpublished data.)

		All Patients.	**P-gp**		**MRP**		**LRP**	
			negative	**positive**	**negative**	**positive**	**negative**	**positive**
n		210	87	123	23	187	67	143
mean OS (overall survival months)		34,5	39,3	31,2	26,9	35,5	37,0	33,4
mean DFS (disease free survival months)		31,4	36,4	27,9	25,6	32,1	33,1	30,6
Mean Age (Years)		60,8	59,0	61,2	63,1	60,0	61,1	59,9
Sex	Male	173	73	100	19	154	51	122
	Female	37	14	23	4	33	16	21
Staging	I	80	38	42	7	73	30	50
	II	32	11	24	9	26	8	27
	IIIa	83	35	43	7	71	23	55
Histology	Spinocellular carcinoma	80	36 (45,00%)	46 (57,50%)	5 (6,25%)	77 (96,25%)	27 (33,75%)	55 (68,75%)
	adenocarcinoma	47	21 (44,68%)	29 (61,70%)	3 (6,38%)	47 (100,00%)	18 (38,30%)	32 (68,09%)
	others	26	8 (30,77%)	19 (73,08%)	1 (3,85%)	26 (100,00%)	6 (23,08%)	21 (80,77%)

Non –Pgp mediated multidrug resistance

Within the past few years a variety of further MDR-associated genes was identified. The discovery of the MDR- associated protein MRP1 was followed by members MRP2 - MRP6 of this transporter family (Cole et al, 1992; Borst et al, 1999) and the lung resistance-related protein (LRP) acting as the major vault protein in the nucleocytoplasmic transport (Scheper et al,1993). They confer the MDR phenotype that is distinct in pattern from mdr1-related resistance, but includes many of the same drugs. MRP belongs to the ABC transporter gene superfamily and operates as an ATP-dependent primary active transporter for substrates conjugated with glucuronide or glutathione (Ishikawa et al, 2000). Overexpression of MRP and LRP is frequently observed in primary NSCLC, especially in the well-differentiated squamous cell carcinomas (Nooter et al, 1996) and expression is significantly higher in NSCLC samples when compared to SCLC samples (Dingemanns et al, 1996).There is also evidence that expression of MRP1 and LRP can be upregulated by sublethal exposure of lung cancer cells to some MDR-related drugs (Berger et al, 2000;Yoshida et al, 2001). Ota et al,

(1995) examined the levels of MRP expression in 104 NSCLC and found that MRP overexpression was associated with a worse prognosis of patients that received postoperative chemotherapy with MRP-related anticancer drugs (vindesine/etoposide). However, the concomitant cooperation of several resistance mechanisms may be often necessary to cause the MDR phenotype. Experimental data indicate that the genes coding for P-gp, MRP and LRP are differentially regulated by extracellular stimuli.

B. Glutathione-dependent enzymes

Another important arsenal that cells utilize to depression of the cytotoxic effects of anticancer drugs are glutathione (GSH) and the GSH-related enzymes glutathione S-transferase (GST) and glutathione peroxidase (GPX). GST catalyzes the conjugation of electrophilic metabolites to GSH to facilitate their excretion. GPX utilizes GSH to remove reactive oxygen intermediates. GSH and its related enzymes are ubiquitously distributed in many normal tissues as well as tumors and are involved in resistance to a wide variety of drugs such as alkylating agents, anthracyclines and vinca alkaloids (Tew, 1994). There is increasing evidence that these enzymes are a determinant factor in the sensitivity of lung tumors to anticancer drugs (Carmichael et al, 1988; Sharma et al, 1993). GST isoenzymes are found in significant amounts in bronchioles and alveoli of normal lung (Awasthi et al, 1987) and most intensely in the bronchial epithelium (Anttila et al, 1993). A number of studies have shown that the amount of GST isoenzymes is even higher in tumors of the lung relative to the surrounding normal tissue (Di Ilio et al, 1988; Clapper et al, 1991). In particular, high levels of glutathionedependent enzymes have been detected in cell lines derived from NSCLC compared to SCLC cell lines (D'Arpa and Liu, 1989). It is speculated that these alterations could account for the differences in drug sensitivity between these tumor types. Evidence that GST may be involved in drug resistance has come from the generation of drug-resistant cell lines in vitro and from transfection studies with GST cDNAs (Nakagawa et al, 1990). Changes in GST expression are most marked in cell lines developed resistant to nitrogen mustard compounds and nitrosoureas and redox cycling drugs such as doxorubicin (Whelan et al, 1989). GST-π isoenzyme is also overexpressed in lung tumors of smokers compared to non-smokers (Volm et al, 1991). It is suggested that GST overexpression may be a part of an adaptive response to enviromental stress to protect against toxic injury. Frequently, a co-expression of P-gp and GST-π is found (Volm et al, 1991; Linsenmeyer et al, 1992). But also a coordinated induction of MRP1 and GSH-related enzymes is reported in malignant cells after exposure to cytostatic agents (Van der Kolk et al,1999). These observations led to the suggestions that these genes share common regulatory mechanisms, and that perhaps a single transcription factor or regulating protein may be involved in their regulation. However, it is unlikely that alterations in the GST are causally related to the development of drug resistance in lung tumors, but rather that co-modification along with other resistancerelated enzymes could mediate drug resistance.

Besides these detoxifying enzymes, normal lung is also efficiently protected against exogenous free radicals by antioxidant enzymes (AOEs). Major human AOE include superoxide dismutases, catalase and enzymes associated with GSH metabolism, all of which are expressed in human lung. In addition, human lung also expresses several thiol-

containing proteins including the families of thioredoxins, thioredoxin reductases and peroxiredoxins. Their expression in human lung is located mainly to alveolar macrophages, bronchial epithelial cells and alveolar epithelium, critical areas in the oxidant protection of human lung (Kinnula et al, 2004). These proteins not only have effects on cell proliferation and cell death, but also protect both non-malignant and malignant cells against radiation and chemotherapy. The redoxregulating proteins are highly expressed in lung tumors (Soini et al, 2001) and are associated with lymph node status and prognosis in NSCLC (Kakolyris et al, 2001). Peroxiredoxins also have effects on the progression and prognosis of lung cancer (Lehtonen et al, 2004).

C. Topoisomerases

In addition to P-gp and non-P-gp-mediated MDR, other mechanisms for resistance to multiple drugs have been described including frequent alterations of topoisomerase II (topo II) activity (Eijdems et al, 1985; Cole et al, 1991). Topo II is an ubiquous nuclear enzyme that is essential for many aspects of DNA function, including replication, recombination and transcription. There is evidence that this enzyme is the target of many clinically important antineoplastic drugs such as anthracyclines, ellipticines, amsacrines and epipodophyllotoxins (D'Arpa and Liu, 1989; Zijlstra et al, 1990). These drugs stabilize the cleavable complex formed between topo II and DNA, resulting in increased DNA excision, detectable as DNA single-strand or double-strand breaks, and DNA-protein cross-links. Drug-induced cell destruction is proportional to the level of topo II. This explains how a reduction in topo II could be a major mechanism of resistance to many antineoplastic drugs. Topo II has also been reported to play a role in cell proliferation. High levels of this enzyme are found in proliferating cells, and very low levels in quiescent cells (Zijlstra et al, 1990). In contrast, there are some reports that increased topo II is associated with resistance to certain DNA-damaging agents (Dingemans et al, 1999). It is also speculated that the increased affinity of topo II for cross-linked DNA in alkylating agents-resistant cells contribute to alkylator resistance by changing DNA topology, thereby facilitating DNA repair (Eder et al, 1995; Pu and Bezwoda, 1999). In surgical tissue samples of primary untreated lung tumors, significant intra- and inter-tumor variation in topo II expression has been observed. Topo II activity is higher in NSCLC as compared to breast cancer (McLeod et al, 1994). On the other hand, topo II activities of SCLC cell lines have been reported to be 2-fold higher than those for NSCLC cell lines, corresponding to their sensitivities to doxorubicin and etoposide (Kasahara et al, 1992). A correlation between topo II gene expression and sensitivity to doxorubicin, etoposide and cisplatin was also found in lung cancer cell lines not selected in vitro for drug resistance (Giaccone et al, 1992). Most lung cancer cell lines selected for resistance to doxorubicin demonstrate decreased levels of topo II expression in addition to P-gp overexpression (Eijdems et al, 1985). Therefore, low levels of topo II expression may predict reduced sensitivity of human lung cancer to several drugs. However, as with mdr1/P-gp expression, this cannot solely explain the drug-resistant phenotype of NSCLC.

D. Metallothioneins

Metallothioneins (MTs) are intracellular proteins of low molecular weight (6-7 kDa) that are present in a wide variety of eukaryotes. MT are characterized by a high content of cysteine and the ability to bind heavy metal ions including zinc, copper, cadmium and platinum. The physiological function of MT is not well understood. Most mammalian tissues contain a basal level of MT, which may vary with the type of tissue. MT has also been demonstrated in a variety of malignancies including colorectal tumors (Öfner et al, 1994), testicular germ cell tumors (Chin et al, 1993) and ovarian tumors (Murphy et al, 1991). MT in lung cancer tissue is significantly elevated when compared to nonmalignant lung tissue (Hartet al, 1993). The synthesis of MT is easily inducible in lung or other organs by certain hormones, cytokines, growth factors, tumor promotors and many other chemicals. Some stressful environmental conditions such as heat, cold and starvation also induce MT (Hamer, 1986). Recently, the synthesis of MT by tumor cells has been proposed as a possible mechanism for the intracellular inactivation of metal-containing chemotherapeutic agents such as cisplatin. MT content and MT mRNA levels correlated well with the sensitivity of SCLC cell lines to cisplatin (Kasahara et al, 1991). A transfected cell line that overexpresses MT proved not only resistant to cisplatin but also resistant to chlorambucil, melphalan and doxorubicin (Kelley et al, 1988). However, cells of various origins selected for cisplatin resistance often, but not always show increased MT expression, suggesting that an increased MT expression alone may not be the sole mediator of cisplatin resistance. Matsumoto et al, (1997) found that the proportion of MT-positive tumors was significantly higher in treated NSCLC compared with untreated NSCLC and treated SCLC, whereas Joseph et al, (2001) demonstrated that MT overexpression was predictive of short-term survival in patients with SCLC undergoing chemotherapy. In a study with human NSCLC, we found a significant relationship between MT expression and doxorubicin resistance in vitro (Mattern et al, 1992). Thus, a number of factors may be involved in the development of drug resistance in lung tumor cells and expression of MT may be one of them.

E. O6-alkylguanine-DNA alkyltransferase

A number of DNA-damaging anticancer agents attack the O6 position on guanine, forming the potent cytotoxic DNA adduct. The DNA repair enzyme O6- alkylguanine-DNA alkyltransferase (ATase), encoded by the gene *MGMT*, repairs alkylation at this site and is responsible for protecting tumor and normal cells from these agents. ATase activity varies widely among different organs, with lung tissue on average lower than others (Citron et al, 1991). However, ATase activity in normal peripheral lung tissue of smokers is significantly higher than that of nonsmokers (Drin et al, 1994; Mattern et al, 1998). Most human lung tumors contain amounts of ATase similar or greater than the tissue from which they originate (Kelley et al, 1988). In SCLC, ATase was found to be significantly lower than in NSCLC, but wide interindividual variations were observed (Oberli-Schrämmli et al, 1994). However, approximately 12% of human lung tumors are deficient in this enzyme (Citron et al, 1993). These ATase-deficient tumors are very

sensitive to the cytotoxic effects of agents that alkylate the O6-position of guanine, such as nitrosoureas (Pegg, 1990).
Thus, evidence suggesting a possible role for ATase in drug resistance of lung tumors comes from the following observations: (1) the level of ATase in tumor cells correlates well with the sensitivity to nitrosoureas (Brent et al, 1985), (2) transfection of the gene for alkyltransferase to ATase-deficient cells decreases the sensitivity to alkylating agents (Kaina et al, 1991), and (3) depletion of the activity of this enzyme by addition of O6-benzylguanine significantly enhances toxicity (Dolan et al,1993).

F. Thymidylate synthase

Thymidylate synthase (TS) plays a central role in DNA biosynthesis and is the target of many chemotherapeutic agents, such as 5-fluorouracil, methotrexate and fluorodeoxyuridine (Washtien, 1982).Moreover, tumor cells resistant to cisplatin and doxorubicin display increased levels of this enzyme (Scanlon et al, 1988; Chu et al, 1991). Human NSCLC strongly express TS in a high percentage of cases (Volm and Mattern, 1992a; 1992b). This expression of TS is significantly related to doxorubicin resistance in vitro and associated with cross-resistance to 5-fluorouracil (Volm et al, 1979). Moreover, TS-positive lung tumors have been noted to be clinically progressive, the affected patients living a significantly shorter time than those with TS negative tumors (Volm and Mattern, 1992a). In addition, evaluation of intratumoral TS activity accurately predicts responsiveness to 5-FU-based chemotherapy in NSCLC patients (Huang et al, 2000; Shintani et al, 2004). However, the relevance of TS relates not only to the importance of this enzyme as a chemotherapeutic target, but also as a DNA synthetic enzyme associated with cell division and proliferation (Stammler et al, 1995;Nakagawa et al, 2004). Nevertheless, high intrinsic levels of TS do not necessarily lead to higher proliferation rates than in cases with low levels of TS (Pestalozzi et al, 1995). A recent study has shown that TS protein binds to *c-myc* mRNA suggesting an involvement in the coordinate regulation of a number of other genes (Chu et al, 1994).

G. Cytochrome P450 enzymes

For the treatment of cancer, the window between drug toxicity and suboptimal therapy is often narrow. Interindividual variation in drug metabolism therefore complicates therapy. Genetic polymorphisms in phase I and phase II enzymes may explain part of the observed interindividual variation in pharmacokinetics and pharmacodynamics of anticancer drugs. The cytochrome P450 superfamily is involved in many drug metabolizing reactions. Information on variant alleles for the different isoenzymes of this family, encoding proteins with decreased enzymatic activity, is rapidly growing. The ultimate goal of ongoing research on these enzymes would be to enable pharmacogenetic screening prior to anticancer therapy. At this moment, potential clinically relevant application of CYP450 pharmacogenetics for anticancer therapy may be found for *CYP1A2* and flutamide *(*Shet et. al. 1997) , *CYP2A6* and tegafur (Dajgo S et. al. 2002), *CYP2C8* and paclitaxel (Dai et. al. 2001).

H. Cell cycle-related proteins

There exists general agreement that cancer chemotherapy is most successful when used on rapidly growing malignant cells (Valeriote and Van Putten, 1975). Experimental data obtained in a variety of systems ranging from mammalian cell cultures to transplanted rodent tumors show that proliferating cells are more sensitive to most cytotoxic agents than are resting cells (Drewinko et al, 1981). These experimental data are supported by the clinical observations that fast-growing tumors usually
respond to treatment, whereas tumors with a low rate of proliferation very often show no response. To estimate the proliferative activity of cancer, various techniques including 3H-thymidine labeling (Alama et al, 1990) or flow-cytometric analysis (Volm et al, 1985; 1988) have been used. Several antibodies have also been produced that label preferentially the nuclei of proliferating (Volm et al, 1995a) and nonproliferating cells (Volm et al,1995b). Although human lung tumors show a wide variation in proliferative activity and tumor doubling times, NSCLC have on average lower labeling indices and longer doubling times than SCLC, perhaps partly accounting for their resistance to cytotoxic drugs (Muggia, 1974; Arai et al, 1994). Moreover, patients whose lung tumors have a high proportion of cells in the S-phase generally die earlier than patients whose tumors have a low proportion of these cells (Alama et al, 1990; Volm et al, 1985; 1988; 1995). Thus, the determination of cell proliferation in clinical material provides a potentially useful marker to estimate sensitivity or resistance to anticancer drugs. Cell proliferation is regulated by both growth-stimulatory and growth-inhibitory proteins (Sherr, 1993). Protein complexes that are composed of cyclins and cyclin-dependent kinases (cdks) are important factors for cellular proliferation. Cyclins are regulatory proteins for cdks and are differentially synthesized and degraded at specific points during the cell cycle (Cordon-Cardo, 1995). Five major classes of mammalian cyclins have been described (cyclin A-E). Cyclin C, D and E reach their peak of synthesis and activity during the G1 phase and regulate the transition from G1 to S-phase. Cyclins A and B achieve their maximum peaks during S- and G2-phases.
We and others could demonstrate that cyclin A expression closely correlates with the proportion of S-phase cells measured by flow cytometry (Volm et al, 1997).

Furthermore, patients with cyclin A-positive lung carcinomas had significantly shorter median survival times than patients with cyclin A-negative carcinomas. A significant correlation between expression of cyclin A and response of NSCLC to doxorubicin in vitro was also detected (Volm et al, 1997).

I. Hypoxia

Because the rate of neovascularization frequently fails to keep pace with tumor growth, tumor vasculature is often inadequate for the tumor mass. Therefore, many solid tumors contain subpopulations of cells that are able to live in hypoxic conditions and are relatively resistant to certain drugs (Teicher, 1994) and irradiation (Höckel et al, 1993). This is partly caused to poor vascularization that reduces the influx of cytostatic agents and lowers the levels of oxygen and nutrients. A growing body of evidence indicates that

cells respond to hypoxic stress by altering the expression of specific genes or proteins (Wilson and Sutherland, 1989; Sutherland et al, 1996). Hypoxia is known to induce one or more transcription factors, the best characterized of which is hypoxia-inducible factor-1 (HIF-1), which in turn stimulates expression of several genes including those involved in drug resistance and endothelial cell growth. Hypoxia-induced resistance to doxorubicin and to methotrexate has been attributed to an amplification of the P-glycoprotein gene and the dihydrofolate reductase gene (Rice et al, 1986; 1987; Luk et et al, 1990; Kalra et al, 1993). Murphy et al, (1994) have recently shown that metallothionein IIA mRNA levels were significantly increased during hypoxia and during reoxygenation. O'Dwyer et al, (1994) investigated the effects of hypoxia on the expression of a group of enzymes involved in drug metabolism. Exposing colon carcinoma cells to hypoxia resulted in a notably increased glutathione content. In a clinical study with NSCLC, it has been shown that poor vascularization, as measured by vessel density, correlates with an upregulation of glutathione s-transferase- π, metallothionein and thymidylate synthase (Koomägi et al, 1995). In another study involving rectal cancer, poor angiogenesis is also linked to an expression of glutathione S-transferase and metallothionein (Mattern et al, 1996). Moreover, lung tumors with low microvessel density and low VEGF expression were more frequently resistant to doxorubicin in vitro than tumors with high microvessel density and high expression of VEGF (Volm et al, 1996). These studies show that hypoxia or poor vascularization result in overexpression of certain detoxicating enzymes which provides an additional insight into cell resistance. Some studies have demonstrated the presence of drug resistance mechanisms in endothelial cells of normal and tumor tissue (Cordon-Cardo et al, 1990; Terrier et al, 1990). The MDR-associated P-glycoprotein and glutathione S-transferase have been localized in normal human endothelial cells and in the stroma of some tumors. Furthermore, Huang and Wright (1994) found that some members of the fibroblast growth factor family, which are potent angiogenic peptides, may mediate resistance to some cytotoxic agents and modify gene amplification properties of tumor cells. Furthermore, there is experimental evidence that tumor cells and vascular endothelial cells within a solid tumor may stimulate each other by paracrine factors (Rak and Kerbel, 1996). On the basis of these studies, it seems reasonable to hypothesize that a highly vascularized tumor may produce elevated levels of angiogenic peptides that induce proliferation of chemoresistant endothelial cells which may confer tumor cell resistance to conventional anticancer therapy.

J. Programmed cell death

The recent progress in the field of biology has indicated that programmed cell death (apoptosis) plays an important role in the chemotherapy-induced tumor cell killing. Since the different antineoplastic agents induce a similar pattern of cell death, it was suggested that a common pathway of apoptosis could exist in the druginduced apoptosis and the defect in the signaling pathway of apoptosis could cause a new form of multidrug resistance in tumor cells. Recent studies in human leukemia cells have demonstrated that chemosensitivity also depends on activation of caspases that are an integral part of the CD95 signaling pathway (Los et al, 1997). Inhibition of caspases not only retarded the

apoptotic process but also provided protection from drug-induced death. In a study with NSCLC, caspase-3 expression correlated with a lower incidence of lymph node involvement and the median survival time was longer for patients with caspase-3-positive tumors than for those with caspase-3-negative tumors (Koomägi and Volm, 2000).
Thus, impairment in the protease effector phase of apoptosis may lead to chemoresistance against several anticancer drugs that is not due to other well-characterized resistance mechanisms such as overexpression of antiapoptotic Bcl-2-related proteins or increased expression of P-gp (Friesen at al, 1997).
Overproduction of Bcl-2, a blocker of apoptosis, prevents cell death induced by nearly most anticancer drugs and radiation, thus contributing to treatment failures in patients with cancer (Miyashita and Reed, 1993; Sartorius and Krammer, 2002). However, several homologs of Bcl-2 have been discovered, some of which function as inhibitors of cell death and others as promoters of apoptosis that oppose the actions of the Bcl-2 protein. Thus, the role of Bcl-2 as a clinically significant prognostic factor of drug resistance remained open (Martin et al, 2003). In a study with 85 human squamous cell lung carcinomas, we found a positive correlation between expression of Bcl-2 and expression of the resistance- related proteins P-gp and GST-π. Moreover, all Bcl-2- positive carcinomas were resistant to doxorubicin in an in vitro predictive test (Volm and Mattern, 1995). These results indicate that Bcl-2 may contribute to drug resistance in NSCLC.

K. Complexity of resistance mechanisms.

During the past few years it has become apparent that multiple mechanisms of resistance play a role in the clinical manifestaion of drug resistance. The study of drug resistance in lung cancer has not identified a single, specific mechanism as a major cause of the resistance observed in a clinical setting. There are now many reports that cell populations exist in human lung tumors which have several resistance mechanisms at once. The parallel assessment of drug resistance parameters in human tumors has shown that individual tumors exhibit different patterns with elevation of none, several or all of the monitored resistance markers . This indicates that each tumor has its own unique resistance factor profile. In lung tumors, Oberli-Schrämmli et al, (1994) observed in a majority of tumors the concomitant overexpression of ATase and GSH-related parameters. In contrast, overexpression of ATase together with P-gp was never observed. There was no correlation between ATase and GSH or its enzymes in colorectal tumors (Redmond et al,1991). However, ATase was frequently co-expressed with other drug resistance parameters in ovarain tumors (Joncourt et al, 1998). An increased expression of P-gp was detected not only concomitant to an overexpression of GST, but also accompanied by a coordinate overexpression of metallothionein and thymidylate synthase in human lung tumor (Volm and Mattern, 1992). A relationship exists between the extent of resistance measured in vitro and the number of detected resistance mechanisms. With an increasing extent of resistance, the number of resistance mechanisms increases (Volm et al,1992). The reasons for the concomitant expression of different resistant mechanisms in human lung tumors are unknown. The increased expression of several resistance markers might be the result of induction of a cascade of resistance- related gene products triggered by chemotherapy or environmental factors. It

was found from in vitro studies that NSCLC of smokers are more frequently resistant and express a higher degree of P-gp and GST-π than tumors of non-smokers (Volm et al, 1991). Thus, smoking may upregulate different detoxifying enzymes, depending on histopathological and clinicopathological variables, to protect the cells from carcinogens but as a consequence render them resistant to drugs. The coordinate expression of different resistance mechanisms in the same tumor may explain why tumors are also resistant to drugs not involved in therapy and why a single marker, e.g. GST-π, may serve as a general marker for resistance and prognosis, irrespective of whether it is itself involved in the resistance mechanism (Mulder et al, 1995).Another explanation for the presence of different resistance mechanisms in human tumors is that tumors are mostly detected at a relatively late stage when they are already large and have metastasized. These tumors are for the most part hypoxic and the vascular network for supply of oxygen and nutrients is substantially lower (Mattern et al, 1996). In fact, it has been shown that various resistance parameters are upregulated in tumors with poor vascularization (Koomägi et al, 1995) and that the reduced vascularization of tumors together with upregulated resistance-related proteins may represent an import
contributing factor to the poor response to chemotherapy and irradiation. There are several hints that detoxifying systems may share common regulatory elements. One possibility is that the resistance factors present in human tumors belong to a set of genes that can be coordinately expressed to protect cells from injury and against different xenobiotics. Many oncogene products are implicated in the regulation of cellular proliferation and, because the growth rate of tumors is an important determinant for the response of tumors to chemotherapy, oncogenes might influence drug resistance by regulation of proliferative activity. It has been reported that c-fos is involved in growth control and cellular differentiation (Verma, 1986). The c-fos protein is associated with the gene product of the proto-oncogene c-jun. The c-fos/c-jun protein complex binds specifically to a DNA sequence referred to as the AP-1 binding site and thereby affects the transcriptional expression of cellular genes (Sassone-Corsi et al, 1988). It has been also demonstrated that the promoter region of the Chinese hamster P-gp gene contains the AP-1 binding site and that this latter is essential for full promoter activity (Teeter et al, 1991). The promoter region of the genomic GST-π also contains an AP-1 motif, which suggests that this gene may be regulated by the cellular oncogenes c-fos and c-jun (Morrow et al, 1989). In a clinical study, surgical specimens of NSCLC of untreated patients were analyzed for expression of c-fos, c-jun and for resistance to doxorubicin. A significant association between drug resistance and expression of c-fos and c-jun proteins was found (Volm, 1993). With a c-fos-transfected cell line it was demonstrated that a ribozyme-mediated decrease in c-fos expression was associated with reduced levels of thymidylate synthase, DNA polymerase β and metallothionein IIA (Scanlon et al, 1991). These results suggest that Fos protein may mediate DNA replication and repair processes through transcriptional activation of the above mentioned genes.

L. Future directions

The recent development of DNA microarray technology for large-scale analyses of gene expression has has a profound impact on biomedical research. Microarrays allow the simultaneous analysis of thousands of genes or proteins in a single experiment. Thus, it is not surprising that the old concept of prediction of drug response and individualized therapy is experiencing a revival. Staunton et al (2001) determined whether the gene expression signatures of untreated cells are sufficient for the prediction of drug sensitivity. Using a panel of 60 human cancer cell lines, gene expression-based classifiers of sensitivity or resistance of 232 compounds were generated. They found that the accuracy of chemosensitivity prediction was considerably better than would be expected by chance. Kudoh et al (2000) used the cDNA microarray to monitor the expression profiles of MCF-7 cells that are selected for resistance to doxorubicin. They found that a subset of genes was constitutively overexpressed in cells selected for resistance to doxorubicin. Ikehara et al, (2004) conducted a study with 47 human lung tumors (using cDNA microarray analysis) to determine whether expression levels of genes were correlated with survival after chemotherapy. They analyzed the expression levels of 1176 genes and found that three genes, G1/S-specific cyclin D2, type II cGMPdependent protein kinase and hepatocyte growth factor-like protein, were significantly correlated with survival. Wigle et al (2002) performed expression profiling on tumor specimens from 39 NSCLC patients and could identify distinct profiles of gene expression correlating with disease-free survival. Significant technological advances in protein chemistry in the last decades have established mass spectrometry as a tool for protein study. The recently developed ProteinChip technology using surface enhanced laser desorption/ionisation (SELDI) mass spectrometry facilitate protein profiling of complex biological mixtures and could be used to discriminate e.g. normal vs. tumor tissues or treated vs. untreated cells. Preliminary results with this technology show that this method could be used to classify and predict histological subgroups as well as nodal involvement and survival in resected NSCLC (Yanagisawa et al, 2003; Zhukov et al, 2003). The promising aspect of all these new methods is the hope that it will be improve the ability to identify those patients who are at high risk of failing therapy.

List of abbreviations used in the text:

Abbreviations: antioxidant enzymes, (AOE); 50% inhibitory concentration, (IC50); called multidrug resistance**,** (MDR); cyclin dependent kinases (cdks); glutathione peroxidase, (GPX); glutathione S-transferase, (GST); glutathione, (GSH); hypoxia-inducible factor-1, (HIF-1); lung resistance protein, (LRP); metallothioneins, (MTs); non-small cell lung cancer, (NSCLC); O6-alkylguanine-DNA alkyltransferase, (ATase); of multidrug resistance-associated protein, (MRP); P-glycoprotein, (P-gp); small cell lung cancer, (SCLC); surface enhanced laser desorption/ionisation, (SELDI); thymidylate synthase, (TS); topoisomerase II, (topo II).

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