Cytokine-mediated reversal of multidrug resistance

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Abstract

The occurrence of the multidrug resistance phenotype still represents a limiting factor for successful cancer chemotherapy. Numerous efforts have been made to develop strategies for reversal and/or modulation of this major therapy obstacle through targeting at different levels of intervention. The phenomenon of MDR is often associated with overexpression of resistance-associated genes. Since the classical type of MDR in human cancers is mainly mediated by the P-glycoprotein encoded by the multidrug resistance gene 1, *mdr1*, the majority of reversal approaches target the expression and/or function of the *mdr1* gene/P-glycoprotein. Due to the fact that the multidrug phenotype always represents the net effect of a panel of resistance-associated genes/gene products, other resistance genes, e.g. those encoding the multidrug resistance-associated protein MRP or the lung resistance protein LRP, were included in the studies. Cytokines such as tumor necrosis factor α and interleukin-2 have been shown to modulate the MDR phenotype in different experimental settings *in vitro* and *in vivo*. Several studies have been performed to evaluate their potential as chemosensitizers of tumor cells in the context of a combined application of MDR-associated anticancer drugs like doxorubicin and vincristine with cytokines. Moreover, the capability of cytokines to modulate the expression of MDR-associated genes was demonstrated, either by external addition or by transduction of the respective cytokine gene. Knowledge of the combination effects of cytokines and cytostatics and its link to their MDR-modulating capacity may contribute to a more efficient and to a more individualized immuno-chemotherapy of human malignancies.

Abbreviations: EGF – epidermal growth factor; FGF – fibroblast growth factor; IFN α , $-\beta$, $-\gamma$ – interferon α , $-\beta$, $-\gamma$; IGF-I, -II – insulin-like growth factor-I, -II; IL-1 α , -1 β , -2, -3, -4, -5, -6 – interleukin-1 α , -1 β , -2, -3, -4, -5, -6; LRP – lung resistance protein; MDR – multidrug resistance; *mdr1* – multidrug resistance gene 1; MRP – multidrug resistance-associated protein; PDGF-AA, -BB – platelet derived growth factor-AA, -BB; PGP – P-glycoprotein; TGF β – tumor growth factor β ; TNF α – tumor necrosis factor α .

Introduction

The occurrence of the multidrug resistance (MDR) phenotype in human tumors – either intrinsically existing or acquired by environmental stress factors – still represents a major obstacle for successful cancer chemotherapy (for review: Roninson, 1991; Clynes, 1994; Gupta and Tsuruo, 1996). The development of the MDR phenotype is often accompanied by elevated expression levels of various so-called MDR-associated genes. Thus, the MDR phenotype represents the net

effect of expression/function of those genes and gene products which are involved in this resistance phenomenon.

Since a distinction has to be drawn between different types of MDR a variety of genes encoding gene products with different regulations, physiological functions, modes of action, and cellular locations have to be expected. For example, the classical MDR type is mainly caused by overexpression of the multidrug resistance gene (*mdr1*) encoding the P-glycoprotein (PGP) which is supposed to act as

an energy-dependent drug efflux pump (Endicott and Ling, 1989; Germann *et al.*, 1993). This protein is capable of extruding certain drugs from cells, leading to decreased drug concentrations within the cell and reduced efficacy of drugs (Chin *et al.*, 1993).

In addition, other genes that are also involved in multidrug resistance phenomenon were identified in cells demonstrating the non-P-glycoprotein-mediated variant of MDR. These genes include for example the gene encoding the multidrug resistance-associated protein (MRP), that is belonging like PGP to the ATP-binding proteins (Cole *et al.*, 1992). Cloning and transfection experiments provided evidence that the MRP gene could confer resistance to a wide spectrum of drugs (Grant *et al.*, 1994). More recently, the gene encoding the lung resistance protein (LRP) has been identified in non-P-glycoprotein expressing cells (Scheper *et al.*, 1993). Analysis of the LRP cDNA sequence led to the identification of the encoded protein as the human major cytoplasmic vault protein suggesting that cytoplasmic vaults are organelles involved in nuclear-cytoplasmic drug transport (Scheffer *et al.*, 1995).

Although several resistance mechanisms are known the failure of chemotherapy protocols by means of MDR-associated cytostatics have usually been attribute to the PGP-mediated MDR phenotype caused by the overexpression of the aforementioned *mdr1* gene. Therefore, the partial or complete inhibition of the *mdr1* gene expression/P-glycoprotein function was, and still is, the major goal in the majority of the studies testing MDR reversal strategies.

There is a variety of strategies to improve the efficacy of a particular cancer treatment regimen aiming at the chemosensitization of chemotherapy-insensitive malignancies in association with the reversal of MDR (Figure I). Besides a variety of MDR circumventing factors, like the reduction of the availability of ATP, liposomal drug encapsulation or the selection of cytostatics unaffected by known resistance-associated proteins, several options exist to modulate the MDR at the different levels of expression and function of the MDR-associated genes/proteins. For example, (i) In the majority of approaches the function of MDR-associated proteins is targeted, e.g. by calcium channel blockers, calmodulin antagonists, cyclosporin, steroids, monoclonal antibodies, inhibitors of PGP-modifying enzymes, etc. (e.g Kellen, 1993; Goldstein, 1995; Ford, 1996; Twentyman and Versantvoort, 1996). Although some of these substances have been examined for reversal of MDR in experimental mod-

els and even in clinical trials, their usefulness remains controversial (e.g. Raderer and Scheithauer, 1993; McLeod, 1996). (ii) To hinder the synthesis of MDR-associated proteins methods like *mdr1*-specific ribozymes (e.g. Scanlon *et al.*, 1994; Kobayashi *et al.*, 1994) and antisense oligonucleotides (e.g. Sola and Colombani, 1996; Cucco and Calabretta, 1996) acting at the translational level were employed. (iii) An alternative approach to reverse the MDR phenotype aims at the expression regulation of MDR-associated genes, e.g. by cytokines. Several studies investigating the expression of MDR-associated genes were performed (Salmon *et al.*, 1989; Scala *et al.*, 1991; Evans and Baker, 1992; Kang and Perry, 1994; Walther and Stein 1994; Licht *et al.*, 1995; Fogler *et al.*, 1995; Walther *et al.*, 1995; Stein *et al.*, 1996a; Stein *et al.*, 1996b; Stein *et al.*, 1997a).

Thus, the participation of a particular gene within the network of resistance-associated genes was evaluated, enabling the definition of the impact of this particular gene on the MDR phenotype. This allows one to estimate whether this gene may represent a target for reversal strategies. Downregulation of the respective gene should consequently lead to a more chemosensitive phenotype enhancing the efficacy of MDR-associated cytostatic drugs. Based on this concept the development of a more sophisticated and individualized chemotherapeutic cancer treatment should be feasible.

Since various cytokines have been most frequently used as chemosensitizing agents in clinical chemotherapy protocols their influence on the MDR phenotype as well as on the expression of MDR-associated genes have been examined by different experimental procedures. Although acting via different signal transduction pathways, some of them cause similar chemosensitizing effects by expression modulation of MDR-associated genes.

In this chapter, reports describing the effects of cytokines as chemosensitizing substances on the expression of resistance-associated genes as well as on the MDR phenotype will be summarized. The mode of application in experimental *in vitro* and *in vivo* models – either by external administration of the cytokine or by transduction of the respective cytokine gene – will be compared. The effects on MDR-associated genes/proteins will be reviewed in this context. Furthermore, clinical trials investigating the beneficial use of these chemosensitizers in combination with MDR-associated cytostatic drugs will be discussed.

Figure 1. Overview of several approaches to reverse/modulate the mdr1/P-glycoprotein-mediated MDR phenotype on different levels of intervention.

Experimental In Vitro Approaches to Modulate the Chemosensitivity of Tumor Cells Towards MDR-Associated Drugs by Cytokines

Within the last two decades more sophisticated approaches for a combination of chemotherapy and immunotherapy have been described. Among a variety of compounds which are able to modulate the MDR phenotype the group of cytokines is gaining more interest. Since it has been shown that tumor response rates may increase when treatment with conventional chemotherapeutic drugs is combined with cytokines (Wadler and Schwartz, 1990), several cytokines were analyzed for their capability to influence the MDR phenotype, and specifically, to modulate chemosensitivity of resistant cells towards MDR-associated anticancer drugs.

A panel of studies analyzing the effects of cytokines with different modes of action were performed

in several experimental settings, comparing their efficacies with varying concentration, time schedule, route of administration, etc. (17 selected studies were summarized in Table I). It appears that cytokines are able to influence or even to reverse the MDR phenotype by enhancing the cytotoxicity of MDR-associated drugs to tumor cells, as demonstrated in 15 of these selected studies. This cytokine-mediated chemosensitization has been analyzed in particular for anthracyclins (Scala *et al.*, 1991; Evans and Baker, 1992; Kikuchi *et al.*, 1992; Monti *et al.*, 1993; Kang and Perry, 1994; Borsellino *et al.*, 1994; Kamikaseda *et al.*, 1994; Walther and Stein, 1994; Licht *et al.*, 1995; Fogler *et al.*, 1995; Stein, *et al.*, 1996a, b), epipodophyllotoxins (Valenti *et al.*, 1993; Morgavi *et al.*, 1995; Ishihara *et al.*, 1995) and vinca alkaloids (Walther and Stein, 1994; Licht *et al.*, 1995; Fogler *et al.*, 1995; Stein *et al.*, 1996a, b). The results were predominantly obtained in human *in vitro*

Table 1. Cytokine effects on the expression of MDR-associated genes and on chemosensitivity of MDR-associated drugs: experimental *in vitro* approaches

Cytokine	Cell line	Gene expression of MDR-associated genes	MDR-associated drug	Chemo-sensitization	Reference
TNF α	leukemia, myeloma	no change in mdrl-mRNA	n.d.	n.d.	Salmon <i>et al.</i> , 1989
IFN α	colon carcinoma	no change in PGP	doxorubicin	+	Scala <i>et al.</i> , 1991
Leukoregulin	myeloma	down regulation in PGP	doxorubicin	+	Evans and Baker, 1992
IL-1 α , -1 β , -2, -3, -4, -5, -6, TNF α , IFN γ , IFN α , EGF, PDGF AA, PDGF BB, IGF I, IFG II, FGF, TGF β		no change in PGP			
TNF α , IFN α , IFN γ	leukemia	n.d.	doxorubicin	+	Kikuchi <i>et al.</i> , 1992
IL-1 α	ovarian carcinoma	n.d.	doxorubicin	+	Monti <i>et al.</i> , 1993
TNF α	ovarian carcinoma	n.d.	VP16	+	Valenti <i>et al.</i> , 1993
IFN α	ovarian carcinoma, hamster	up regulation in mdrl-mRNA up regulation in PGP	doxorubicin	-	Kang and Perry, 1994
IL-1 α , TNF α	leukemia, mouse melanoma	n.d.	doxorubicin	+	Borsellino <i>et al.</i> , 1994
TNF α	glioma	n.d.	actinomycin D, daunomycin, doxorubicin, mitomycin C	+	Kamikaseda <i>et al.</i> , 1994
TNF α , IL-2, IFN γ	3 colon carcinoma lines	down regulation in mdrl-mRNA	doxorubicin, vincristine	+	Walther and Stein, 1994
	1 colon carcinoma line	no change in mdrl-mRNA			
TNF α	glioblastoma	n.d.	VP16, VM26	+	Morgavi <i>et al.</i> , 1995
TNF α	mesothelioma	no change in PGP	doxorubicin, vindesine	+	Licht <i>et al.</i> , 1995
IFN α (+MRK16)	colon and breast carcinoma	no change in PGP	vincristine, doxorubicin, paclitaxel	+	Fogler <i>et al.</i> , 1995
IFN β	glioblastoma	n.d.	VP16	+	Ishihara <i>et al.</i> , 1995
TNF α , IL-2,	colon carcinoma	down regulation in mdrl-mRNA down regulation in PGP	doxorubicin, vincristine	+	Stein <i>et al.</i> , 1996a
TNF α	murine tumor cell line	n.d.	actinomycin D	+	Lasek <i>et al.</i> , 1996
TNF α	colon carcinoma	down regulation in LRP-mRNA down regulation in LRP-protein up regulation in MRP-mRNA	doxorubicin, vincristine	+	Stein <i>et al.</i> , 1997a

models, established either from solid (e.g. colon carcinoma, ovarian carcinoma, glioblastoma) or from hematopoietic cancers. In the majority of the cases an elevated accumulation of fluorescent PGP substrates, like rhodamine 123 or doxorubicin, was measured after cytokine treatment reflecting the sensitization of the tumor cells. To determine whether cytokines may have an impact on chemosensitivity MTT or XTT assays, as well as comparative analyses of viable vs. dead dyed (e.g. trypan blue or crystal violet) cells, were performed.

A modulation or even a reversal of the MDR phenotype was achieved with a variety of different cytokines (Table I). Interestingly, among others the tested panel included cytokines such as tumor necrosis factor α (TNF α), interferons α , β , and γ (IFN α , β , and γ), and several interleukins known to belong to cytokine groups acting via different regulatory pathways. In our experiments the highest modulating effects were obtained mainly by using TNF α , either in combination with doxorubicin or together with vincristine as observed in colon carcinoma and in glioblastoma cell lines (Walther and Stein, 1994; Walther *et al.*, 1995; Stein, *et al.*, 1996a; Stein *et al.*, 1997a). Besides the effects on chemosensitivity this cytokine also enhanced the accumulation of fluorescent drugs more dramatically than, for example, IL-2 or IFN γ . In this context, an important aspect may also be considered: the participation of P-glycoprotein itself in the transport of certain cytokines. Based on the UIC2 shift assay performed in peripheral blood lymphocytes there are evidences that e.g. IL-2, IL-4 as well as IFN γ , but not TNF α , were effluxed (Raghu *et al.*, 1996; Drach *et al.*, 1996; Park and Mechetner, 1998).

These consistent results describing the cytokines' capability to reverse the MDR phenotype (Table I) are even more surprising since the treatments with the various cytokines were carried out using different cytokine concentrations (mostly ranging up to 1000 U/mL) as well as different time schedules, ranging from several minutes (e.g. 15 min) to several days (up to 72 h). The rapid effects caused by the cytokines within 15 min and 4 h were investigated in 3 studies (Evans and Baker, 1992; Licht *et al.*, 1995; Lasek *et al.*, 1996a). In some reports the cytokine effect on chemosensitivity towards MDR-associated drugs was analyzed at only one time point, usually between 18 and 24 h (5/17; Salmon *et al.*, 1989; Valenti *et al.*, 1993; Kang and Perry, 1994; Kamikaseda *et al.*, 1994; Morgavi *et al.*, 1995). Another set of ex-

periments examined cytokine effects after long term incubations spanning up to 48 or even 72 h (8/17; Scala *et al.*, 1991; Kikuchi *et al.*, 1992; Monti *et al.*, 1993; Borsellino *et al.*, 1994; Walther and Stein, 1994; Ishihara *et al.*, 1995; Stein, *et al.*, 1996a; Stein *et al.*, 1997a). In only 7 reports were more than one time point included in the study design for obtaining longitudinal data of cytokine-mediated effects (Evans and Baker, 1992; Kikuchi *et al.*, 1992; Walther and Stein, 1994; Fogler *et al.*, 1995; Stein *et al.*, 1996a; Lasek *et al.*, 1996a; Stein *et al.*, 1997a). Thus, the comparison of these data is very difficult thereby complicating the evaluation of the results in general. However, the combination of cytokines and drugs is apparently of advantage for gaining a more sensitive phenotype of tumor cells, despite this great variety in experimental conditions.

Another factor may influence the efficacy of such a combination treatment, namely: simultaneous or sequential exposure of the cells to cytokines and drugs. From our observations, the pretreatment of cells with the respective cytokine is a very decisive prerequisite for obtaining chemosensitization of the cells. Simultaneous administration of cytokine and drug had no, or only marginal, effect on the MDR phenotype. Therefore, the sequential administration of cytokine and drug in accordance with a specific time schedule will provide the most promising approach for a cytokine-mediated reversal of the MDR phenotype.

We analyzed the cytokine-induced/mediated modulation of the chemosensitivity to MDR drugs, of the function of PGP and of the expression pattern of MDR-associated genes, e.g. in a panel of colon carcinoma cells. The extent of the cytokine-mediated modulation was dependent on the expression patterns of MDR-associated genes. This holds true for pairs of cell lines comparing the parental line versus the different multidrug resistant sublines selected by exposure to different concentrations of anticancer drugs.

This fact may reflect that not necessarily all cells of all types will respond to this kind of combination therapy, or at least, they will respond to a different degree. Having a potential clinical application in mind, it demonstrates the necessity of an individualized treatment regimen considering the resistance parameters of each patient to gain the most advantageous therapeutic effect.

Experimental In Vitro Approaches to Modulate the Expression of MDR-Associated Genes by Cytokines

Although the fact that cytokines are able to modulate the MDR phenotype is frequently observed and widely accepted, their effects on the expression of MDR-associated genes, especially of the *mdr1* gene, have been controversial. Within the panel of the selected reports the effect of the respective cytokines on *mdr1* expression at the mRNA and/or protein level was investigated in only 8 out of 17 studies. In 6 studies no modulation of *mdr1* and/or PGP expression was observed, even if a chemosensitizing effect was achieved by exposure to several cytokines or growth factors (TNF α , Salmon *et al.*, 1989; IFN α , Scala *et al.*, 1991; IL-1 α , -1 β , -2, -3, -4, -5, -6, TNF α , IFN γ , IFN α , EGF, PDGF AA and BB, IGF I and II, FGF, TGF β , Evans and Baker, 1992; TNF α , IL-2, IFN γ , Walther and Stein, 1994; TNF α , Licht *et al.*, 1995; IFN α , Fogler *et al.*, 1995).

A modulated *mdr1* gene expression was observed in 4 studies (Table I): downregulation of PGP (leukoregulin for up to 2 h; Evans and Baker, 1992), upregulation of *mdr1*/PGP (IFN α for 24 h, Kang and Perry, 1994), downregulation of *mdr1* (TNF α , IL-2 and IFN γ for 48 and 72 h, Walther and Stein, 1994), and downregulation of *mdr1*/PGP (TNF α , IL-2 for 48 h, Stein *et al.*, 1996a). Interestingly, those studies demonstrating decreased *mdr1* gene expression after cytokine exposure correlated with a chemosensitization to the MDR-associated anticancer drugs doxorubicin and vincristine; the only study demonstrating enhanced *mdr1* gene expression at both expression levels (in a hamster derived cell line) described the failure of chemosensitization after cytokine (IFN α) treatment (Kang and Perry, 1994).

When evaluating these results it has to be considered that all of these studies were carried out in different experimental settings using various qualitative and quantitative methods to determine mRNA or protein expression levels by means of different detection systems (primers, probes, monoclonal antibodies, controls). More importantly, the selection of cytokines and drugs and their treatment conditions (concentrations, incubation times, application scheme) were not standardized and are difficult to compare. Studies verifying the cytokine-caused effects on *mdr1* gene expression at both expression levels were performed in only two laboratories.

An additional complication for MDR reversal strategies, in general, is the existence of other, non-P-glycoprotein-mediated mechanisms of resistance that may or may not be amenable to a given reversal approach. Concurrent operation of at least two distinct resistance mechanisms has been observed in a single tumor cell population (e.g. Hasegawa *et al.*, 1995; Brock *et al.*, 1995; Filipits *et al.*, 1996). Furthermore, the frequent occurrence of as many as three overlapping phenotypes of multidrug resistance (*mdr1*, MRP, LRP) was observed in a large panel of human tumor cell lines (Izquierdo *et al.*, 1996). In a series of human mammary carcinoma cell lines expression of *mdr1*, MRP and LRP was also observed, in the parental lines as well as in the multidrug resistance sublines (Stein *et al.*, 1997b). Thus, the MDR phenotype always represents the net effect of the expression of a variety of genes which contribute to development of the MDR phenotype.

Therefore, it has to be taken into account that each cytokine may influence not only one target gene to cause the reversal of the MDR phenotype. In this context, monitoring of a panel of MDR-associated genes which may be affected by a certain cytokine could be valuable. Thus, we were interested in investigating the generality of the effects of for example TNF α or IL-2 in influencing *mdr1* gene expression (Table I; Stein *et al.*, 1996a) with respect to the other MDR-associated genes, LRP and MRP (Stein *et al.*, 1997a). After long term exposure of resistant cells to both cytokines we found a downregulation of *mdr1* mRNA and P-glycoprotein, verified by enhanced doxorubicin accumulation and increased chemosensitivity to doxorubicin and vincristine. After examining the influence of TNF α on the expression of LRP and MRP, anti-complementary effects were observed: while LRP expression was downregulated in TNF α -treated colon carcinoma cell lines at both expression levels, MRP expression was upregulated in the cell line with the lower resistance (HCT116) and was unaffected in HCT15 cells possessing high resistance. Since functional and chemosensitivity assays were performed in these studies, they do correlate with the decreased *mdr1* and LRP expression, but not with the modulation of the MRP gene. These results underline the complexity of the MDR phenomenon making reversal approaches even more difficult.

To interpret these data at the molecular level basic knowledge about the promoter regions of these resistance genes has to be considered. It seems likely that the effects of TNF α or IL-2 described herein

may result from the activation/deactivation of a signal transduction/transcription factor cascade (e.g. Osborn *et al.*, 1989; Raj *et al.*, 1994). However, while considerable information is available about the *mdr1* gene promoter (e.g. Ueda *et al.*, 1987; Ogura *et al.*, 1992; Goldsmith *et al.*, 1993; Uchiumi *et al.*, 1993; Stein *et al.*, 1994; Combates *et al.*, 1994; Bargou *et al.*, 1997) and some information has more recently become available about the MRP gene promoter (Zhu and Center, 1994; Zhu and Center, 1996), nothing is currently known regarding the LRP gene promoter. However, since specific elements responding to environmental stress stimuli have been identified within the *mdr1* promoter and several consensus sequences for binding of transcription factors – e.g. of the TNF α -induced cascades – have been described for the *mdr1* and the MRP promoter regions (Zhu and Center, 1994; Zhu and Center, 1996), the same might be hypothesized for the LRP gene promoter. Furthermore, elevated expression levels of these genes were determined in several experimental systems which were previously exposed to MDR-associated drugs, underlining these observations. Based on the knowledge of cytokine-induced pathways and/or transcription factors specifically designed analyses have to be performed addressing the question whether a cytokine-modulated expression of MDR-associated genes is a causal or an accompanying event for the reversal of MDR.

Experimental In Vitro Approaches to Modulate the MDR Phenotype and/or the Expression Pattern of MDR-Associated Genes by Transduction of Cytokine Genes

Numerous investigators have reported that cytokines are able to potentiate drug activity in multidrug resistant cell populations. Thus, the inclusion of cytokine administration in combination-chemotherapy protocols may provide a clinical approach to reverse multidrug resistance. However, this approach is complicated by the toxic side effects associated with systemic administration of cytokines (e.g. for TNF α : Schiller *et al.*, 1991; Sidhu and Bollon, 1993). To avoid the disadvantages of systemic cytokine administration the direct introduction of expression vectors encoding the respective cytokine gene into tumor cells might be an alternative strategy to deliver cytokines in tumor vicinity.

We tested the feasibility of this approach by transducing the human genes for TNF α (Walther *et al.*,

1995; Stein *et al.*, 1996b; Stein *et al.*, 1997a) and for IL-2 (Stein *et al.*, 1996b) into human multidrug resistant tumor cells. Cytokine expression was driven by the cytomegalovirus promoter. The effects of gene-transduced and secreted TNF α or IL-2 on the expression of MDR-associated genes like *mdr1*, MRP and LRP were examined (Table II).

In initial experiments, analyzing the effects of transduced TNF α on MDR in human glioblastoma cells (Walther *et al.*, 1995), we found that secreted TNF α is able to potentiate chemosensitivity towards MDR-associated drugs. This observation was associated with an increased accumulation of rhodamine 123 within the transduced cells, correlating to a decrease in P-glycoprotein.

In a second set of experiments, the capability of transduced and secreted cytokines to modulate the MDR phenotype as well as the expression and function of MDR-associated genes/proteins was investigated in human colon carcinoma cells known to possess high intrinsic MDR levels, thus complicating chemotherapy with MDR-associated anticancer drugs. Thus, colon carcinoma cells with different intrinsic degrees in MDR phenotype were transduced with either a retroviral vector harboring the human TNF α gene or with a retroviral vector carrying the human gene for IL-2. Cell clones which demonstrated high amounts of the respective secreted cytokine were chosen for analysis. As determined for both cell lines (HCT15, HCT116), the expression and release of TNF α as well as of IL-2 caused a decreased expression of the *mdr1* gene, as detected at both the mRNA and the protein level. This finding was confirmed by enhanced accumulation of the P-glycoprotein substrate doxorubicin. Enhanced chemosensitivity towards doxorubicin and vincristine was observed in the cytokine transduced cell clones. The most striking effects were measured in those cytokine transduced cell clones with the highest amounts of the secreted cytokine. Comparison of the two cytokines suggested that TNF α was a more powerful modulator of *mdr1* expression than IL-2 (Stein *et al.*, 1996b).

In the next step we analyzed whether expression of the MDR-associated genes MRP and LRP was affected by transduction of the cytokine genes encoding TNF α or IL-2 into HCT15 and HCT116 colon carcinoma cells (Stein *et al.*, 1997a). For TNF α , anti-complementary effects on the expression of these two genes were found, paralleling the data determined by external addition of the cytokine. Thus, LRP gene expression was found to be down regulated in TNF α

Table 2. Effects of transduced cytokines on the expression of MDR-associated genes and on chemosensitivity of MDR-associated drugs: experimental *in vitro* approaches

Cytokine	Cell line	Gene expression of MDR-associated genes	MDR-associated drug	Chemo-sensitization	Reference
TNF α	glioblastoma	down regulation in PGP	doxorubicin, vincristine	+	Walther <i>et al.</i> , 1995
TNF α , IL-2	colon carcinoma	down regulation in mdrl-mRNA down regulation in PGP	doxorubicin, vincristine	+	Stein <i>et al.</i> , 1996b
TNF α	colon carcinoma	down regulation in LRP-mRNA down regulation LRP-protein up regulation in MRP-mRNA up regulation in MRP-protein	doxorubicin, vincristine	+	Stein <i>et al.</i> , 1997a
IL-2	colon carcinoma	no change in LRP-mRNA no change in MRP-mRNA	doxorubicin, vincristine	+	Stein <i>et al.</i> , 1997, unpublished data

secreting cells of both lines, apparently dependent on the amount of the secreted cytokine. In contrast, MRP gene expression was found to be enhanced in TNF α expressing clones of both lines, even if the more resistant one (HCT15) was not affected by the external TNF α application. Therefore, it might be possible that transduction of cytokine genes and subsequent release of cytokines by transduced tumor cells could mimic the long term external cytokine treatment, leading to effective multidrug resistance reversal in these cells.

Furthermore, we have found that IL-2 was also able to reduce the expression of mdrl at mRNA and protein levels (Stein *et al.*, 1996b). But, using the same experimental conditions, IL-2 did not affect LRP or MRP expression at the mRNA or protein level in these colon carcinoma cell lines.

In contrast to our findings regarding the cytokine-mediated mdrl modulation, some studies report no modulation of mdrl gene expression at either the mRNA or protein levels, neither after external cytokine addition (e.g. Safrit *et al.*, 1993) nor in cytokine-transduced cells. The question has to be addressed why the observed cytokine effects are consistent concerning the final chemosensitization capability of certain cytokines, but differ for their mdrl expression modulation capacity. Since these results may have an important impact for the design of clinical combination treatments of immunotherapy and chemotherapy based on molecular knowledge, standardized *in vitro* and *in vivo* experiments should be performed (same experimental systems, same cytokines and drugs in comparative concentrations and

time schedules, same methods and detection systems, etc).

To analyze cytokine-mediated effects which finally modulate the phenotype of MDR it has to be considered that their influence on resistance-associated genes may affect only one facet within the interplay of the entire cell. This pleiotropy of cytokine effects had been investigated in view to a variety of different other aspects. In this context, for TNF α for instance, influences on tumor cell differentiation (Kikuchi *et al.*, 1992), tumor growth (Walther *et al.*, 1993), metastasis potential (Kreuser *et al.*, 1995), apoptosis (Flugy *et al.*, 1995; Malorni *et al.*, 1996), induction of proteins such as topoisomerase I and II (Kreuser *et al.*, 1995) or mitochondrial manganese superoxide dismutase (Zyad *et al.*, 1994) were observed. Molecular signal transduction pathways implying the key events for the molecular decision which cytokine-caused effect will be realized are currently extensively discussed.

However, the finding of the diverse modulation of different MDR-associated genes by external stimuli also observed by others (Komarov *et al.*, 1997) point to the fact that MDR reversal strategies should not only focus on the presence/modulation of the mdrl/P-glycoprotein, but should also consider that an entire panel of resistance-associated genes might be involved which are possibly regulated in contrasting ways.

Experimental In Vivo Approaches to Modulate the Chemosensitivity Towards MDR-Associated and Non-MDR-Associated Drugs by Cytokines

To evaluate the data of cytokine-caused modulation obtained by means of *in vitro* systems this therapeutic approach was also examined in some experimental settings in mice (Table III). The verification of the *in vitro* data by *in vivo* experiments is even more important for a translation of this combined immunotherapy and chemotherapy into practice.

Within the last decade additive or even synergistic antitumor effects of cytokines such as TNF α and anticancer drugs have been observed in mice (Regenass *et al.*, 1987; Krosnick *et al.*, 1989). Interestingly, the greatest enhancements of chemosensitivity were again achieved by a defined sequential administration of the cytokine (pretreatment) and the anticancer drug demonstrated by using the murine sarcoma model. Moreover, these chemosensitizing effects were not exclusively described for cytokine combinations with MDR-associated drugs like doxorubicin, but also for combinations including cyclophosphamide or 5-fluorouracil, which do not belong to the MDR-drug family. This underlines that cytokines, on the one hand, may affect the expression/function of a variety of resistance-associated genes which confer resistance to non-identical spectra of cytostatic drugs; on the other hand, they may act via different mechanisms besides those of gene expression modulation.

The beneficial employment of immuno-chemotherapy was also described for IL-2 or IFN α in combination with cyclophosphamide using murine sarcoma and carcinoma models (Kedar *et al.*, 1992). The most efficacious regimen was achieved by the combination of all three substances, i.e. the two cytokines with the drug. There was evidence that the sequential administration of these cytokines might be of advantage compared to concurrent administration.

To analyze the chemosensitizing potential of TNF α with regard to the anticancer drugs actinomycin D and cyclophosphamide, a murine leukemia model was employed (Lasek *et al.*, 1995). The combination of chemotherapy and immunotherapy, including all agents, produced a beneficial effect which resulted for example in a significant prolongation of survival of leukemia-bearing mice. This study also indicates the potential of appropriate combinations of cytotoxic drugs with cytokines against neoplasia. In murine melanoma models studies with TNF α and IFN γ in combination with actinomycin D had been carried out

(Lasek *et al.*, 1996). After testing the influence of the cytokines or the drug alone, the highest synergistic effect was described for the combination of the two cytokines, TNF α and IFN γ , with the anticancer drug. This enhancement of cytotoxicity was achieved by local as well as systemic application. Since this combination had displayed higher efficacy than either treatment alone the feasibility of a combination of immunotherapy and chemotherapy had been verified.

Taken together, the hypothesis of a more efficient combination treatment of immuno- and chemotherapy drawn from the *in vitro* approaches has been confirmed in different *in vivo* models. Nevertheless, analyses of these effects at the molecular level are still lacking. This fact should encourage further investigations studying the influences of MDR- versus non-MDR-drugs in combination with different cytokines. Results about altered expression and/or function of MDR-associated genes/proteins could possibly unveil the molecular basis for a specific combination regimen. Thus, studies designed to understand the molecular basis may provide a useful prerequisite for a more sophisticated immuno-chemo-therapy of cancer patients.

Clinical Trials and Future Perspectives

Chemotherapy of human malignancies is frequently performed as combination therapy of cytostatics and substances known to improve their effectiveness, such as cytostatics with different modes of action (polychemotherapy), anti-estrogens, monoclonal antibodies, lymphokine-activated killer cells, tumor-infiltrating lymphocytes, cytokines, etc.

A huge panel of trials described the feasibility of protocols with immuno-polychemo-therapy in accordance to the response rates; but these rates may differ greatly which might mainly be due to different individual patient inclusion/exclusion criteria, variations in treatment schedules, and differences between single and multicenter studies. However, the majority of these studies demonstrated a more efficient cancer therapy by combining cytostatics and cytokines which may be probably caused by chemosensitizing effects due to the respective cytokine.

Although these treatment regimens were not designed to analyze the molecular basis of the combination effects of cytokines and cytostatics they do probably target at the modulation of the expression/function of MDR-associated genes/proteins as well. Thus, trials carried out to examine the effects of cytokine-drug-

Table 3. Combination of immunotherapy and chemotherapy in mice: experimental *in vivo* approaches

Experimental system	Cytokine	Anticancer drug	Chemo-sensitization	Reference
Meth A sarcoma	TNF α	adriamycin, 5-fluorouracil, cyclophosphamide	+	Regenass <i>et al.</i> , 1987
MCA 106 sarcoma	TNF α	cyclophosphamide, doxorubicin, 5-fluorouracil	+	Krosnick <i>et al.</i> , 1989
MCA-105 sarcoma, M109 carcinoma	IL-2, IFN α	cyclophosphamide	+	Kedar <i>et al.</i> , 1992
L1210 leukemia	TNF α	cyclophosphamide, actinomycin D	+	Lasek <i>et al.</i> , 1995
MmB16 melanoma	TNF α , IFN γ	actinomycin D	+	Lasek <i>et al.</i> , 1996

combinations on the MDR phenotype at the molecular level are urgently needed. To achieve the administration of an individualized therapy, the inclusion of molecular resistance parameters accompanying those clinical trials might provide useful additional informations for the selection of the appropriate cytokine and drug combination.

Assuming that this concept of a cytokine-caused chemosensitization can be feasible treating in previously multidrug resistant tumors, the application of cytostatics, which belong to the MDR-family and which were until now inefficient prior to the cytokine treatment, might be of advantage. Thus, a new kind of cytokine-cytostatics combination will become useful with a view to treating certain tumors.

In summary, based on the development and the improvement of cancer chemotherapy in the last two decades, a new level of a more sophisticated treatment regimen including strategies for MDR reversal is strongly needed for current and future cancer therapy design. Since molecular knowledge has been rapidly growing within the last few years, new opportunities for the translation of this knowledge into the clinical practice become accessible. However, to use these possibilities for reasonable combinations of anticancer drugs and cytokines, comparable clinical trials investigating the molecular mechanisms are desired: (i) to evaluate the resistance status of each individual patient; (ii) to define the resistance patterns of certain cancers; (iii) to compare specific combination protocols of certain cytokines with certain cytostatic drugs with respect to chemosensitization *and* with respect to modulated gene expression/function of MDR-associated genes/proteins; (iv) to prove the correlation of the molecular data on resistance with response rates of the tumors; and finally, (v) to design patient-specific

immuno-chemo-therapy protocols. The performance of this kind of clinical trials in association with molecular analyses of MDR-associated parameters may contribute to the final goal of a more *efficient* and a more *individualized* therapy of human cancers.

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