Advances in Brief

Rapid Activation of *MDR1* Gene Expression in Human Metastatic Sarcoma after *in Vivo* Exposure to Doxorubicin[1]

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Abstract

Overexpression of P-glycoprotein (Pgp), a multidrug transporter encoded by the *MDR1* gene, is associated with chemoresistance in some human solid tumor malignancies. To date, analyses of *MDR1* levels in solid tumors have examined constitutive increases in expression at relapse. In the present study, we have evaluated the acute induction of *MDR1* gene expression in a solid human tumor as a function of time in response to *in vivo* exposure to chemotherapy. Five patients with unresectable sarcoma pulmonary metastases underwent isolated single lung perfusion with doxorubicin. Relative *MDR1* gene expression was measured in metastatic tumor nodules and normal lung specimens after initiation of chemoperfusion. In four of five patients, a 3–15-fold (median, 6.8) increase in *MDR1* RNA levels was detected in tumors at 50 min after administration of doxorubicin. In contrast, normal lung samples had very low levels of *MDR1* RNA prior to perfusion, and no acute increases were observed after therapy. These findings demonstrate, for the first time, that *MDR1* gene expression can be rapidly activated in human tumors after transient *in vivo* exposure to cytotoxic chemotherapy.

Introduction

The development of MDR[4] in cancer patients represents a major obstacle to successful cancer chemotherapy. Although acquired MDR is a well-documented clinical phenomenon, the molecular events mediating *in vivo* tumor response and subsequent emergence of tumor chemoresistance are presently ill defined. Of the many models of drug resistance studied, overexpression of Pgp, encoded in humans by the *MDR1* gene, is frequently implicated in the acquisition of the MDR phenotype in human cancer cell lines (reviewed in Refs. 1 and 2). Increased levels of Pgp are thought to confer resistance to tumor cells by decreasing net intracellular accumulation of a variety of structurally and functionally unrelated antineoplastic agents, including anthracyclines (*e.g.*, doxorubicin), *Vinca* alkaloids, epipodophyllotoxins, and antibiotics. Despite the large body of evidence in support of the role of Pgp overexpression in MDR *in vitro*, the clinical relevance of *MDR1* gene expression has not been clearly elucidated. Although there is good evidence for a correlation between *MDR1* expression and chemoresistance in tumors of the hematopoetic lineage (3), a multitude of retrospective clinical studies have demonstrated a wide spectrum of *MDR1* RNA and protein levels in solid tumor malignancies (1, 2). Furthermore, several reports have identified *MDR1* gene overexpression as an independent negative prognostic factor in clinical outcome (4–7). However, a major limitation in the design and interpretation of these conventional clinical studies has been the time lag between the administration of chemotherapeutic agents and the determination of *MDR1* status. In other words, these studies represent static measurements of gene expression and have not addressed the immediate molecular events occurring in the *in situ* tumor bed in response to acute drug exposure. Therefore, in light of recent studies demonstrating acute activation of the *MDR1* gene *in vitro* in response to chemotherapy (8–11), we have evaluated the increase in *MDR1* gene expression *in vivo* during acute drug exposure. Five patients with sarcoma pulmonary metastases received an acute course of high-dose doxorubicin delivered via an ILP circuit. Using a semiquantitative reverse transcription-PCR method, we measured *MDR1* RNA levels in normal lung and metastatic tumor nodules of these patients immediately before and after chemoperfusion. This study represents the first attempt to examine the possibility that *MDR1* gene expression is rapidly activated after acute *in vivo* exposure of a human solid malignancy to cytotoxic chemotherapy.

Materials and Methods

Patients and Tissue Procurement. Between June 1995 and February 1997, five patients with sarcoma pulmonary metastases underwent ILP with doxorubicin as part of an ongoing Phase I trial at Memorial Sloan-Kettering Cancer Center. This Institutional Review Board-approved protocol enrolls patients with pathologically documented metastatic sarcoma to the lung, not amenable to resection, to receive organ-specific cytotoxic chemotherapy via an isolated perfusion circuit. Fig. 1 is a schematic representation of the perfusion technique. Through a left thoracotomy, the left main pulmonary artery and both pul-

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[4] The abbreviations used are: MDR, multidrug resistance; MDR1, multidrug resistance-1; Pgp, P-glycoprotein; ILP, isolated single lung infusion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

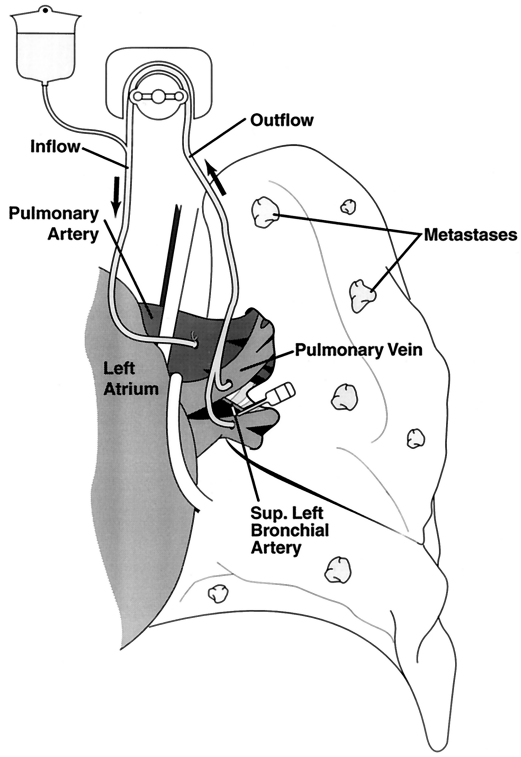

Fig. 1 Schematic representation of the isolated lung perfusion circuit. *Sup.*, superior.

monary veins were isolated, proximally occluded, and individually cannulated. Just prior to the initiation of the doxorubicin perfusion (*i.e.*, time 0), tumor and normal lung wedge biopsies were obtained. The doxorubicin solution was then delivered through the pulmonary artery at a rate of 500–1000 ml/min for a period of 10–20 min, followed by a 10-min wash-out with buffered Hespan (6% hetastarch in 0.9% sodium chloride). Additional tumor and normal lung biopsies were obtained at 20 and 50 min after the initiation of the doxorubicin chemoperfusion. The procured tumor nodules were trimmed from adjacent normal tissue, frozen immediately by immersion in liquid nitrogen, and stored at −70°C. Normal lung specimens were preserved separately in a similar manner. At the completion of the wash-out phase, the vessels were decannulated, the arteriotomy and venotomies were repaired, and all clamps were removed to reestablish pulmonary circulation. Subsequently, the chest incision was closed in a standard fashion. Clinical tumor response was evaluated radiographically by postoperative computed tomography of the chest.

Tissue Doxorubicin Measurements. A portion of each frozen sample was processed for measurement of tissue doxorubicin levels as follows (12). The tissue was homogenized by a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) in 0.02 M KH_2PO_4 buffer (pH 3.8; 1:5 w/v). Forty μl of internal standard (daunorubicin, 10 mg/liter; Cerubidine; Wyeth Laboratories, Philadelphia, PA), 30 μl of KH_2PO_4, 60 μl of 33% silver nitrate, and 390 μl of 80% acetonitrile in water were added to 300 μl of tissue homogenate. Samples were vortexed and centrifuged for 10 min at 3000 rpm to precipitate proteins. Fifty-μl aliquots of the supernatant were analyzed by high-performance liquid chromatography using a Waters pump series 510 and an Autosampler 717 (Waters Corp., Milford, MA). Detection was performed with a Waters 470 scanning fluorescence detector at an excitation wavelength of 480 nm and an emission wavelength of 560 nm. The stationary phase was Zorbax Phenyl (3 mm) packed in a stainless-steel tube (6-mm inside diameter × 40-mm length). The mobile phase was 28% acetonitrile in 0.34 M phosphoric acid solution at a flow rate of 1.0 ml/min. Data were collected and calculated using the Millennium version 2.10 program (Waters Corp.) and expressed as the ratio of the peak area to that of the internal standard. All assays were performed in duplicate and reported as mean concentrations.

Quantitation of *MDR1* Gene Expression by Reverse Transcription-PCR Assay. Frozen tissue specimens were pulverized in a steel mortar situated on a bed of liquid nitrogen. Total cellular RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (13). *MDR1* mRNA levels were quantitated using the technique described by Horikoshi *et al.* (14) Briefly, each RNA isolate was reverse-transcribed into cDNA using random hexanucleotide primers. Serial dilutions of the newly synthesized cDNA (3-fold dilutions for *MDR1*; 10-fold dilutions for standards) were then used as substrates for independent PCR amplification of *MDR1* and internal standards (β-*actin* and *GAPDH*). The PCR primers were synthesized by Integrated DNA Technologies, Inc.(Coralville, IA). Each 5′ primer contained the T7 polymerase promoter sequence 5′-TAATACGACTCACTATA-3′, linked to a transcription initiation sequence (the next four to six bases), which functioned to improve the yield of the amplified gene fragment ~500 fold when the PCR product was transcribed with T7 polymerase. The primer sequences for *MDR1* and β-*actin* genes used in these experiments have been reported previously (15). The 5′ and 3′ primers for GAPDH were T7–5′-GGGACATCTCTGCCCCCTCTGCTG-3′ and 5′-CCCTCCGACGCCTGCTTCAC-3′, respectively. PCR amplification and T7 RNA polymerase transcription were performed as described previously (14). [α-^{32}P]CTP-labeled transcription products were electrophoresed on a 6% denaturing polyacrylamide gel containing 8 M urea. The gels were dried and exposed to film. The bands representing the products of *MDR1*, β-*actin*, and GAPDH amplification were excised and counted in a liquid scintillation counter. The linear amplification range for *MDR1* and the internal standards was established for each of the cDNA samples. Relative gene expression was calculated by determining the ratio between the amount of the radiolabeled PCR product within the linear amplification range of *MDR1* and the internal standard genes. Each cDNA was subjected to a minimum of two independent PCR analyses. Although the absolute numbers obtained from duplicate or triplicate experiments sometimes differed, the relative numbers (*i.e.*, fold activation) were always similar.

Results

Table 1 summarizes the clinicopathological and dose-response characteristics of the five patients. Primary histologies

Table 1 Patient clinicopathological and dose-response characteristics

Patient	Age (yr)	Sex	Primary diagnosis	Site	Prior chemotherapy	Dose of doxorubicin delivered via ILP (mg/m^2)	Clinical response
1	30	F	Synovial cell sarcoma	Thigh	MAID[a]	40	Progression of disease
2	46	F	Uterine leiomyosarcoma	Uterus	Doxorubicin and DTIC	40	Progression of disease
3	29	F	Clear cell sarcoma	Paraspinal	None	40	Progression of disease
4	26	F	Alveolar soft part sarcoma	Thigh	MAID	40	Progression of disease
5	44	M	Malignant fibrous histiocytoma	Thigh	MAID	80	Progression of disease

[a] MAID, Mesna, Adriamycin, ifosfamide, and DTIC (dacarbazine).

Table 2 Mean tissue concentrations of doxorubicin (mg/gm) after isolated lung perfusion

	Normal lung		Tumor	
Patient	20 min	50 min	20 min	50 min
1	[a]	[a]	[a]	[a]
2	8.89	0.58	0.77	[a]
3	10.56	4.64	10.56	5.03
4	31.48	10.1	3.54	6.62
5	63.90	57.34	44.54	33.54

[a] Insufficient tissue for high-performance liquid chromatography assay.

included one visceral, one truncal, and three extremity soft tissue sarcomas. Four of the five patients (patients 1, 2, 4, and 5) had received prior treatment, with doxorubicin as a major component of the chemotherapeutic regimen. On the basis of a dose escalation protocol, the first four patients underwent lung perfusion with doxorubicin at 40 mg/m^2; the fifth patient received the 80 mg/m^2 dose. The most recent computed tomography evaluations of the chest have revealed progression of disease in the perfused lung of all five patients.

To verify the effectiveness of ILP as a drug delivery system, tissue levels of doxorubicin were measured using high-performance liquid chromatography; microgram amounts of doxorubicin were detected in the normal lung and tumor specimens obtained at various times after initiation of chemoperfusion (Table 2). The general trend for the lower tissue doxorubicin levels at 50 min is most likely a result of the Hespan wash-out, during which time most of the unbound drug was cleared from the pulmonary microcirculation.

Quantitative analysis of the mRNA isolates from untreated tumor samples (*i.e.*, time 0) in the first four patients revealed a 3-fold variation in baseline expression of the *MDR1* gene when normalized to the expression of a β-*actin* control (Fig. 2); patient 5 had no detectable *MDR1* RNA levels prior to perfusion. At 20 min after initiation of doxorubicin perfusion, a noticeable rise in the *MDR1*:β-*actin* mRNA ratio was observed in the first four patients (Fig. 2*A*). Moreover, this rise was further augmented and sustained at 50 min after doxorubicin exposure, at which time *MDR1* RNA was also detected in the tumor specimen from patient 5. To assure that the relative differences measured were not a consequence of changes in expression of the control β-*actin* gene, *MDR1* RNA levels were also normalized to the expression of a second control gene, *GAPDH*. Although there was some variation in the absolute levels of activation when normalized against each standard gene, essentially the same pattern of *MDR1* gene activation was noted (Fig. 2, *A* and *B*). It is important to note that in normal lung specimens, which contained very low intrinsic levels of *MDR1* transcript, no acute increase in *MDR1* mRNA levels was observed during or after chemoperfusion (data not shown). Fig. 2*D* depicts the distribution of the fold increases in relative *MDR1* gene expression as a function of time after exposure of the metastatic tumor nodules to doxorubicin. A median 4- and 6.8-fold increase in *MDR1* RNA levels was observed within 20 and 50 min, respectively, after the initiation of chemoperfusion.

Discussion

The overexpression of genes associated with the development and maintenance of drug resistance phenotypes has been attributed to the selection and subsequent clonal expansion of preexisting drug-resistant cells (16) Although several drugs, by virtue of their mutagenic properties, have been suggested to increase the frequency with which these subpopulations arise, the possibility that chemotherapeutics may actually induce drug-resistant variants has remained largely unexplored. However, a number of recent studies indicating that the expression of the *MDR1* gene can be rapidly and transiently induced in cultured cell lines exposed to a variety of chemotherapeutic agents (8–11) prompted us to investigate the possibility that development of *de novo* clinical chemoresistance may be, at least in part, mediated by direct induction of *MDR1* gene expression by antineoplastic agents.

Patients with sarcoma pulmonary metastases demonstrated acute up-regulation of *MDR1* mRNA in response to a transient *in vivo* course of chemotherapy. It is interesting to note that all five patients had progression of disease, despite high-dose chemoperfusion. Although lack of clinical response in this Phase I trial cannot be solely attributed to the observed increase in *MDR1* expression, our findings are consistent with a clinical correlation between *MDR1* overexpression and tumor chemoresistance. It should also be noted that, unlike what has been suggested by some *in vitro* studies, previous exposure to chemotherapy is not a prerequisite for gene activation, because patient 3 had not received prior treatment, yet demonstrated a similar induction of *MDR1* expression. Therefore, the possibility exists that acute activation of *MDR1* expression may play a role in both intrinsic and acquired drug resistance.

The mechanism underlying the rapid increase in *MDR1* mRNA levels in human tumors during chemotherapy has yet to be investigated. Because steady-state levels of mRNA are de-

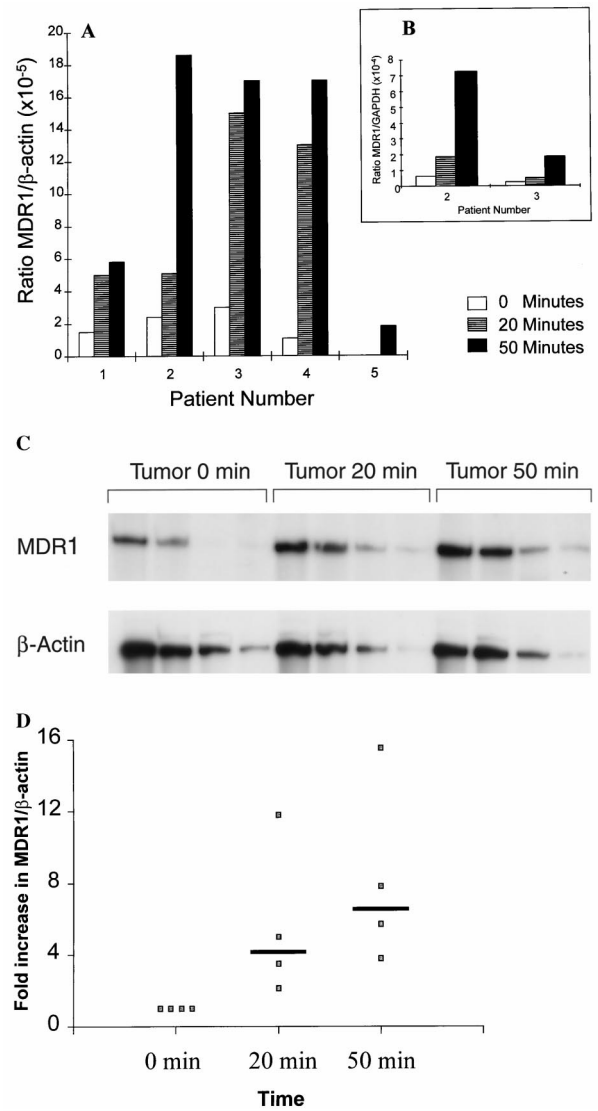

Fig. 2 Time-dependent changes in the relative *MDR1* gene expression in tumor samples of the patients undergoing isolated lung perfusion with doxorubicin. *A*, *MDR1* gene expression relative to β-actin. The PCR products are 157 and 232 bp, respectively. *B*, *MDR1* gene expression relative to GAPDH. *C*, representative autoradiogram of labeled *MDR1* and β-*actin* PCR products of cDNA serial dilutions (3-fold dilutions for *MDR1*; 10-fold dilutions for β-*actin*) at 0, 20, and 50 min after tumor exposure to doxorubicin. The data shown are from patient 2. *D*, scatterplot of the distribution of the fold increases in *MDR1*:β-*actin* ratio in the tumor nodules of those patients showing *MDR1* gene induction. *Thick black bar*, median value at each time point.

termined by both the rate of synthesis and the rate of degradation, a change in either of these two pathways could account for the observed increase in gene expression. However, several observations lead us to favor transcriptional activation of the *MDR1* promoter as the target of chemotherapeutic challenge: (*a*) countless studies of gene expression have indicated that regulation at the level of gene transcription is the primary means by which cells rapidly respond to changes in their environment; (*b*) a number of studies in cultured cell lines have indicated that the *MDR1* promoter can be activated by a variety of stress inducers, including heat shock, heavy metals, differentiating agents, and chemotherapeutic agents (reviewed in Ref. 17). Indeed, we have shown recently that the transcription factors NF-Y and SP1 and the transcriptional coactivator P/CAF mediate activation of the *MDR1* promoter by UV and chemotherapeutics (18); and (*c*) the relatively long half-life of *MDR1* mRNA in cultured cells suggests that an increase in mRNA stability in response to drug challenge would not result in a notable increase in *MDR1* mRNA levels within the time course of our studies. We are presently investigating the possible mechanisms of activation of the *MDR1* gene by chemotherapeutics both in cultured cells and in an equivalent *in vivo* rodent model of isolated lung perfusion (19).

The clinical implications of our findings are far-reaching. Although the surgical operative time limitations of our model did not allow an extended evaluation of the course of *MDR1* gene activation and subsequent measurements of Pgp synthesis, corresponding *in vitro* studies indicate that induction of *MDR1* mRNA levels by chemotherapeutic agents is most often accompanied by increases in Pgp protein levels (9). Moreover, this activation is reversible, and expression decreases over time after drug removal (9). If this is true in the clinical setting, it suggests that alternative drug delivery schedules and the use of chemosensitizing agents could potentially abrogate the tumor response in favor of the host. Although the initial Phase I trials of Pgp reversal agents have had limited clinical success because of suboptimal tissue concentrations and significant dose-limiting toxicities, the new generation of MDR chemomodulators, including cyclosporin A and its potent analogue PSC 833, have been shown to prevent induction of a number of genes, including *MDR1* (20). With a more detailed molecular understanding of *MDR1* gene regulation, particularly with respect to the cellular factors involved in *MDR1* gene activation in response to chemotherapeutic challenge, these new resistance modifiers may assume a greater role in the armamentarium of the clinical oncologist.

This study represents the first observation of a rapid induction of *MDR1* gene expression in a solid human tumor in response to chemotherapeutic exposure. Additional studies are required to determine the generality of this effect, with respect to both chemotherapeutic agent and tumor type, as well as the kinetics of *MDR1* RNA and protein induction and its impact on therapeutic outcome. Nevertheless, this observation has the potential to create a critical shift in our approach to the evaluation of *MDR1* gene expression in clinical samples and suggests that tumor types in which a clear correlation between *MDR1* expression and chemoresistance has not been demonstrated should be reevaluated for a possible role of acute, and possibly transient, *MDR1* induction in clinical response. Clarification of the precise mechanisms involved in *MDR1* gene activation should allow us to prevent or effectively modulate tumor chemoresistance, which will translate into improved clinical response.

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