In Vitro Analysis of the Relationships Between Metallothionein Expression and Cisplatin Sensitivity of Non-small Cellular Lung Cancer Cells

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Abstract. *Background: Cisplatin-based therapy is a pivotal type of chemotherapy for non-small cell lung cancer (NSCLC) and chemoresistance to cisplatin represents one of the most significant barriers to improving long-term clinical outcomes. Materials and Methods: The present study aimed at examining metallothionein (MT) expression in six NSCLC cell lines as well as examining effects of exposure to cisplatin on MT expression in the most cisplatin-resistant (97/97) and the cisplatin-sensitive (DV90) cell lines. Results: The most cisplatin-resistant NSCLC cell line [97/97; (IC_{50})=4.659 μM] exposed to the highest concentration of cisplatin (10 μM) exhibited decreased nuclear MT expression (MTn=6) compared to cells cultured in medium with a lower concentration of cisplatin (0, 1 and 5 μM) (MTn=12). A higher cytoplasmic metallothionein expression (MTc=6) was found in the 97/97 cell line exposed to the highest concentration of cisplatin (10 μM), compared to cells cultured in the medium with lower concentrations of cisplatin (0, 1 and 5 μM) (MTc=3). The most cisplatin-sensitive NSCLC cell line (DV90; IC_{50}=0.184 μM) was characterized by a significant decrease of both nuclear and cytoplasmic MT expression with increasing cisplatin concentrations (5 vs. 10 μM). Conclusion: Nuclear and cytoplasmic expression of MT has no significant impact on the development of cisplatichemoresistance in NSCLC cell lines. The present study suggests that cisplatin resistance in NSCLC is metallothionein-independent.*

Non-small cell lung cancer (NSCLC) is still a major cause of morbidity and mortality in European countries, with a 5-year survival rate from time of diagnosis of around 11% in Europe (1, 2). The management of NSCLC requires a multi-disciplinary treatment, including a combination of surgery, radiotherapy and chemotherapy, depending on the clinical stage and overall performance status (3). Cisplatin-based chemotherapy is a important component of NSCLC treatment for all stages of the disease, especially for patients with clinically-advanced disease (3, 4). For these patients, cisplatin-based chemotherapy is a critical point in determining their survival and improving their quality of life. Metallothioneins (MTs) are low-molecular weight proteins which form four basic families. As pleiotropic cellular proteins, MTs are involved in cell proliferation and apoptosis, binding of metal ions, scavenging of free radicals, and resistance to certain chemotherapeutics (*e.g.* cisplatin) (5, 6). Unfavourable prognostic significance of MT-1 and MT-2 has been widely discussed for many types of human malignancies, such as breast (7, 8), ovarian (9), colon (10), and pancreatic cancer (11), and soft tissue tumours (12).

In patients with NSCLC, recent studies revealed that up-regulation of *MT-1F* and *MT-2A* mRNA predicted for poor patient survival (13). Interestingly, the same group showed that shorter survival of these patients was associated with high expression of MT-1/2 and Ki-67 in neoplastic cells measured by immunohistochemistry (14).

In our previous work, cisplatin-resistant A2780RCIS ovarian carcinoma cells were exposed to increasing cisplatin concentrations, and the subcellular expression of MT (isoforms 1 and 2) was determined by immunocytochemistry

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(15). The studies demonstrated that cisplatin-resistant A2780RCIS cells exposed to cisplatin typically manifested nuclear MT expression. The study also demonstrated that exposure to cisplatin was paralleled by increased MT expression in cell nuclei. The nuclear expression of MT was also found to be specific for ovarian cancer with poor clinical outcome. No relationship was demonstrated between cytoplasmic expression of MT and clinical variables (15).

In vitro studies revealed the strict relationship between expression of MT and resistance to cisplatin (1, 16). Based on previous studies, there appear to be no single mechanism explaining chemoresistance to therapy exhibited by NSCLC (17, 18). One possible explanation of this biological phenomenon includes direct interaction between MTs and cisplatin, in which a single molecule of MT was found to be capable of binding ten molecules of cisplatin (19). Moreover, MTs may potentially inhibit apoptosis, and thus provide resistance to anti-neoplastic agents. The third mechanism which can protect cancer cells from chemotherapeutic agents is free radical scavenging by MTs (6).

In the present study, using immunocytochemistry, we examined the relationship between MT expression, its subcellular localization and chemosensitivity of NSCLC cells to different concentrations of cisplatin.

Materials and Methods

Cell lines and cell culture. We studied the following human NSCLC cell lines: 97/97, 117/96, 51/96 (primary tumour cell lines of lung adenocarcinomas established in our laboratory derived from patients with primary cancer who had surgery for NSCLC at the Charité University Hospital); and A427/97, Ben/97 and DV-90 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Human carcinoma cells were grown in Leibovitz L-15 medium (Biowhittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum (FCS) (Gibco/BRL, Grand Island, NY, USA), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l $NaHCO_3$, 1% minimal essential vitamins and 20,000 kIE/l trasylol in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell proliferation assay. Chemoresistance was tested using a proliferation assay based on sulphorhodamine B (SRB) staining, as described previously (20, 21). Briefly, 800 cells per well were seeded in triplicates, in 96-well plates. After 24 h attachment, cisplatin (GRY-Pharm, Kirchzarten, Germany) was added at different concentrations for a 5-day incubation, before SRB staining was performed. The half maximal inhibitory concentration (IC_{50}) values were calculated from three independent experiments for each cell line.

Additionally, the most cisplatin-resistant NSCLC cell line, 97/97, was cultured for three days on microscope slides in the presence of the following concentrations of cisplatin: 0, 1, 5, and 10 μM (GRY-Pharm, Kirchzarten, Germany). The experiments were run in triplicates.

Immunocytochemistry. Immunostaining of MT was performed using the complete panel of lung carcinoma cell lines, as described previously (15). Studied cells were grown on microscope slides and fixed in ice-cold methanol–acetone mixture (1:1) for 10 min. After rehydration, immunostaining reactions were performed using the monoclonal mouse antibodies (clone E9) detecting isoforms 1 and 2 of MT (DakoCytomation, Glostrup, Denmark) at 1:100 dilution in Antibody Diluent, Background Reducing (DakoCytomation). The cells were incubated with the antibody for 1 h at room temperature. Subsequently, they were incubated with biotinylated antibodies (15 min, room temperature) and with the streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP; DakoCytomation). NovaRed (Vector Laboratories, Peterborough, UK) was used as a chromogen, employing 10 min incubation at room temperature. All the slides were counterstained using Meyer's haematoxylin. Every reaction was accompanied by the negative control in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation).

Scoring of immunostaining results. Intensity of the immunohistochemical reactions was appraised using the semi-quantitative immunoreactive score (IRS), in which intensity of the reaction and the percentage of positive cells were scored. The final result represents the product of scores given nuclear MT (MTn) and cytoplasmic MT (MTc) and ranges between 0 and 12 (22) (Table I). The intensity of immunohistochemical reactions was evaluated independently by two pathologists. In cases of divergence, the evaluation was repeated using a double-headed microscope.

Results

Cisplatin sensitivity of the studied cell lines. The sensitivity of the various human NSCLC cell lines to treatment with cisplatin was determined by the assessment of IC_{50} values (Table II). The most sensitive cell line, DV90, exhibited an IC_{50} of 0.184 μM, and the most cisplatin-resistant cell line, 97/97, had an IC_{50} of 4.659 μM.

MT immunostaining in the studied cell lines. In the case of MT immunostaining, nuclear and cytoplasmic localization of MT of different intensities in individual cases were obtained (Table II, Figure 1). Strong nuclear metallothionein expression (IRS MTn=12) was found in the 97/97 and 117/96 cell lines. Both these NSCLC cell lines were chemoresistant to cisplatin. Low nuclear metallothionein expression (IRS MTn=2) was observed in the Ben/97 cell line, which was cisplatin sensitive.

On the other hand, strong cytoplasmic metallothionein expression (IRS MTc=12) was found in the chemosensitive NSCLC cell line DV90 and low cytoplasmic metallothionein expression was mainly observed in cisplatin-resistant cell lines: 97/97 (IRS MTc=3) and 117/96 (IRS MTc=1).

MT expression and cisplatin incubation. In the case of the most cisplatin-resistant NSCLC cell line (97/97; IC_{50}=4.659 μM), a decrease in nuclear MT expression (IRS MTn=6) was found in cells exposed to the highest concentration of cisplatin (10 μM) as compared to cells cultured at lower concentrations (IRS MTn=12). Moreover, we demonstrated higher cytoplasmic

Table I. *Evaluation criteria of MT expression using the immunoreactive score (IRS) (22).*

Percentage of positive cells	Points	Intensity of reaction	Points
None	0	None	0
<10%	1	Weak	1
10-50%	2	Moderate	2
51-80%	3	Intense	3
>80%	4		

Table II. *Chemosensitivity to cisplatin and metallothionein expression in cell lines.*

Cell line	IC_{50} (μM)	IRS	
		MTc	MTn
117/96	2.460	1	12
51/96	2.152	8	3
97/97	4.659	3	12
A 427/97	1.544	1	4
Ben/97	0.440	9	2
DV90	0.184	12	8

MTc: Cytoplasmic expression of metallothionein; MTn: nuclear expression of metallothionein; IC_{50}: half-maximal inhibitory concentration; IRS: immunoreactive score.

metallothionein expression (IRS MTc=6) in cells exposed to the highest concentration of cisplatin (10 μM) as compared to these cultured at lower concentrations of cisplatin (IRS MTc=3) (Table III, Figure 2).

The most cisplatin-sensitive NSCLC cell line (DV90; IC_{50}=0.184 μM) was characterized by the significant decrease of nuclear and cytoplasmic MT expression with increasing cisplatin concentrations (from 5 to 10 μM) (Table III, Figure 3).

Discussion

In the present study, we have described the divergent patterns of MT expression in six NSCLC cell lines, examined by immunocytochemistry. Additionally, cell lines were exposed to increasing concentrations of cisplatin and the influence on subcellular expression of MT was analyzed.

Cisplatin-based therapy is a pivotal type of chemotherapy for NSCLC. Additionally, cisplatin is usually given in combination with taxanes (paclitaxel, docetaxel), camptothecin analogs (irinotecan, topotecan), gemcitabine, and vinca alkaloids (vinorelbine, vincristine) (3). Cytotoxic activity of cisplatin is mediated by its interaction with DNA to form DNA adducts, which activate several signal transduction pathways, and result in activation of apoptosis

Table III. *Cisplatin exposure and expression of MT in the most chemoresistant NSCLC cell line [97/97; (IC_{50})=4.659 μM)] and in the most chemosensitive NSCLC cell line [DV90; (IC_{50})=0.184 μM)].*

Cell line	Cisplatin (μM)	IRS	
		MTc	MTn
97/97	0	3	12
	1	3	12
	5	3	12
	10	6	6
DV90	0	12	8
	1	12	8
	5	12	8
	10	6	2

(23). There are several mechanisms responsible for cisplatin resistance, which represents one of the most significant barriers to improving long-term survival in NSCLC patients. Cisplatin resistance mechanisms include: increased inactivation by thiol-containing molecules *e.g.* MTs and GSTs; reduced intracellular drug accumulation (caused by the activation of anti-neoplastic agents efflux); dysfunction of tumor-suppressor p53; modulation of DNA damage repair pathways; and overexpression of apoptosis inhibitors (24).

The data on the relationship between MT expression and resistance to cisplatin are divergent. Our *in vitro* study showed that the most cisplatin-resistant NSCLC cell line, 97/97, when exposed to 10 μM of cisplatin demonstrated a decrease in nuclear MT expression compared to cells cultured at lower concentrations of cisplatin.

Matsumoto *et al*. investigated the influence of cisplatin-based chemotherapy on MT expression in NSCLC specimens using immunohistochemistry (25). In untreated tumors, 27% (15/56) of NSCLCs stained positively. In treated tumours, 80% (12/15) of NSCLCs stained positively. The proportion of positively-stained tumors was significantly higher in treated NSCLC compared with untreated NSCLC (p=0.0005). Our results are contrary to theirs, they indicated MT expression increased following chemotherapy, and such expression may confer resistance in lung cancer, especially NSCLC. These divergent results may be associated with different experimental models; we used an *in vitro* method to investigate the impact of MT expression on cisplatin resistance in NSCLC cell lines, in contrast to Matsumoto *et al*.'s study which was based on immunohistochemical analysis using tissue material from patients with NSCLC.

In vitro analysis of a cisplatin-resistant ovarian carcinoma cell line (A2780RCIS) exposed to cisplatin typically manifested nuclear MT expression (15). The study also demonstrated that exposure to cisplatin was paralleled by increased MT expression in cell nuclei (15). A possible

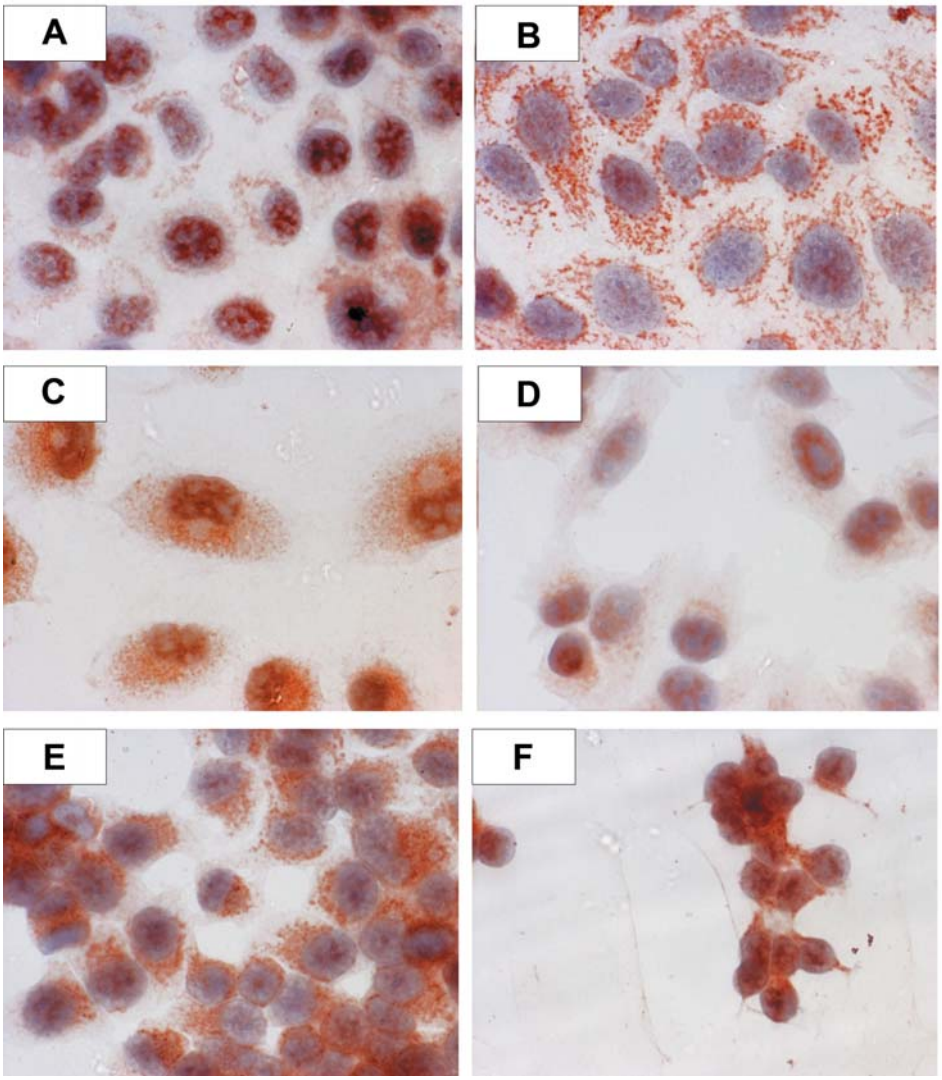

Figure 1. *Immunocytochemical localization of metallothionein expression in the studied cells (×400; haematoxylin). A: 117/96, Note strong nuclear and weak cytoplasmic staining. B: 51/96, Note weak nuclear and medium cytoplasmic staining. C: 97/97, Note strong nuclear and weak cytoplasmic staining. D: A 427/97, Note medium nuclear and no cytoplasmic staining intensity. E: Ben/97, Note weak nuclear and strong cytoplasmic staining. F: DV90, Note the strong nuclear and strong cytoplasmic staining.*

explanation of this biological phenomenon relates to the existence of a different mechanism of cisplatin-resistance in NSCLC, an MT-independent one.

Summing up, in the present study, we showed that nuclear and cytoplasmic expression of MT has no significant impact on the development of cisplatin resistance in NSCLC cell lines. With increasing cisplatin concentrations, we observed a reduction of nuclear expression of MT in the most cisplatin-resistant NSCLC cell line, 97/97, and in the most cisplatin-sensitive NSCLC cell line, DV90. Our study suggests that in NSCLC, another metallothionein-independent mechanism of cisplatin resistance exists.

Conflicts of Interest

The Authors have no conflicts of interest.

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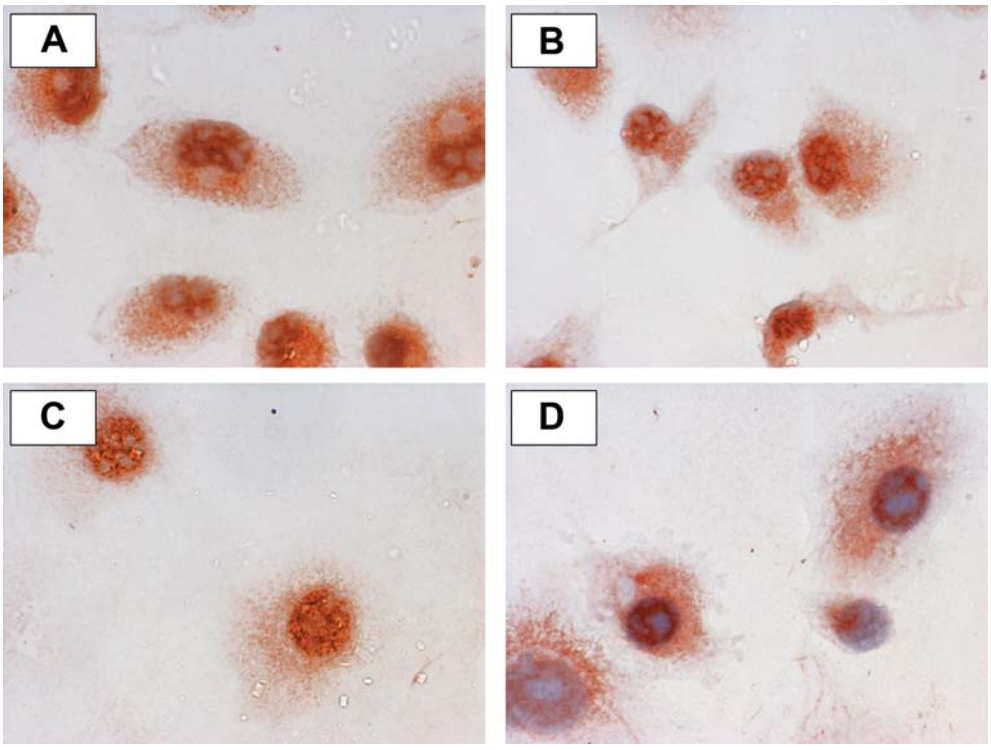

Figure 2. *Metallothionein expression in the 97/97 cell line treated with 0 (A); 1 (B); 5 (C) and 10 (D) μM of cisplatin (×600; haematoxylin).*

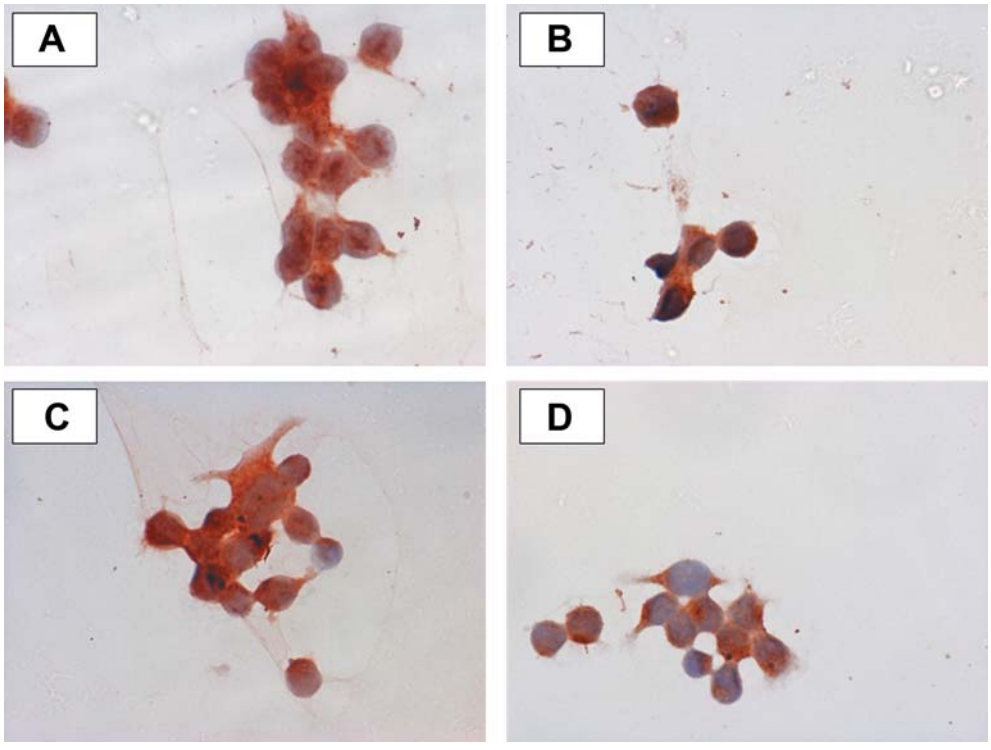

Figure 3. *Metallothionein expression in the DV90 cell line treated with 0 (A); (B) 1; 5 (C) and 10 (D) μM of cisplatin (×600; haematoxylin).*

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