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MECHANISM OF INTERACTION BETWEEN CISPLATIN AND HUMAN RECOMBINANT INTERFERON GAMMA IN HUMAN OVARIAN-CANCER CELL LINES

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Human ovarian carcinoma cells (2008 and its cisplatin-resistant sub-line 2008/C13*) were sensitized to cisplatin by treatment with human recombinant gamma interferon (IFNγ). IFNγ produced no significant change in the uptake of CDDP. Exposure of 2008 and 2008/C13* cells to IFNγ resulted in a time-dependent decrease of cellular glutathione and total glutathione-S-transferase activity, principally the π isoform. By contrast, the treatment of 2008 and 2008/C13* cell lines with IFNγ induced rather than suppressed metallothionein II_A mRNA levels. IFNγ changed neither the formation of total platinum-DNA adducts, nor DNA repair. A significant decrease in c-*erb*B-2 expression was observed both in sensitive and in resistant cell lines after treatment with IFNγ, and this decrease was dose-dependent. Our results indicate that the mechanism of IFNγ-induced sensitization in human ovarian-cancer cell lines is multifactorial.

Ovarian carcinoma is one of the leading causes of cancer death in women. Cisplatin (CDDP) is the most commonly used agent for the treatment of this disease. Unfortunately, the clinical use of CDDP is limited by its toxic profile and by the frequent development of resistance (Andrews and Howell, 1990).

Recently, the incorporation of biological agents, often called biological response modifiers (BRM), in combination with standard chemotherapeutic agents offers a challenge to medical oncologists, since the assumptions of their use differ with the chemotherapeutic agents used. While the mechanism of the anti-tumor activity of human recombinant gamma interferon (IFNγ) is not known, it is now well established that IFNγ has direct anti-tumor properties both *in vitro* and *in vivo* (Saito *et al.,* 1986; Malik *et al.,* 1991). There have been few studies of the interaction of IFNγ with cisplatin *in vitro:* Marth *et al.* (1989) showed a synergistic effect with A2780 and HTB-77 ovarian cell lines and only an additive effect with OVCAR-3 ovarian cell lines. The combination of cisplatin and IFNγ has been shown to be highly synergistic, as demonstrated by median-effect analysis, in a CDDP-sensitive ovarian-cancer cell line (2008) and its 10-fold-CDDP-resistant sub-line (2008/C13*) (Nehmé *et al.,* 1994). Moreover, we demonstrated that IFNγ sensitized the cytotoxic effect of CDDP.

Five different biochemical mechanisms have been identified that can influence the sensitivity of cells to CDDP (Andrews and Howell, 1990; Timmer-Bosscha *et al.,* 1992): (1) impairment of cellular uptake (Andrews *et al.,* 1988*b*); (2) elevation of glutathione content or glutathione-S-transferase activity (Andrews *et al.,* 1988*a;* Chen *et al.,* 1989); (3) elevation of metallothionein (Andrews *et al.,* 1987); (4) variations in DNA repair (Eastman and Schulte, 1988); (5) modification of proto-oncogene expression (Hancock *et al.,* 1991).

In this study, we investigated the effects of IFNγ on the different steps of cellular pharmacology of CDDP on an ovarian-cancer cell line, 2008, and its 10-fold-CDDP-resistant sub-line, 2008/C13*.

MATERIAL AND METHODS

Drugs, chemicals, enzymes and molecular reagents

Recombinant human IFNγ was obtained from Boehringer-Mannheim (Meyland, France). Cisplatin was obtained from R. Bellon (Nevilly, France).

The RPMI-1640 medium and the fetal bovine serum (FBS) were purchased from Seromed (Nunc, Roskilde, Denmark). The epidermal growth factor (EGF) was from Boehringer-Mannheim. 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), 4-chloronaphtol, NADPH, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH reductase were purchased from Sigma (St. Louis, MO). ^{3}H-thymidine was from Amersham (Les Ulis, France) and [α-^{32}P]dCTP was from Du Pont de Nemours (Les Ulis, France).

Cell lines and cell cultures

Two human ovarian-cancer cell lines were used in this study: 2008 and its 10-fold-cisplatin-resistant sub-line (2008/C13*) were generously provided by Dr. S.B. Howell (University of California, San Diego, La Jolla, CA).

Cell lines were maintained in RPMI-1640 medium supplemented with 5% (v/v) FBS, insulin (5 μg/ml) and EGF (2 ng/ml) and kept at 37°C in a humidified atmosphere containing 5% CO_2.

Platinum accumulation

For CDDP accumulation studies, cells growing in log phase were harvested and plated onto plastic tissue-culture flasks (Nunc, Roskilde, Denmark) (75 cm^2) at a density of 6.10^6 cells/flask. Twenty-four hours later, the cells were treated with cisplatin for 1 hr at concentrations that produced synergy with IFNγ, in the presence or absence of IFNγ concentrations defined by the fixed ratio of concentrations of the 2 drugs used for median effect analysis in our previous work (Nehmé *et al.,* 1994). The IFNγ concentrations used were: 8 and 20 U/ml for the 2008 cell line, 40 and 80 U/ml for 2008/C13*. The cells were harvested by trypsinization following drug treatment, counted and centrifuged; the final pellet was reconstituted with H_2O and disrupted by sonication; the platinum concentration was determined in the sample by flameless atomic absorption spectrophotometry.

Protein extraction and Western-blot analysis

Cells growing in log phase were treated with IFNγ for 0 to 72 hr at concentrations that produced synergy with CDDP; these concentrations were up to the IFNγ concentration, which produced inhibition of 50% growth of cells (IC_{50}). The IC_{50} for the 2008 and 2008/C13* cell lines were 20 and 80 U/ml respectively. At different time intervals (4, 12, 24, 48, 72 hr), cells were harvested by scrapping in extraction buffer (10 mM

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Tris, pH 7.4/1.5 mM EDTA) containing the protease inhibitor leupeptine (50 μg/ml). Cells were then disrupted by sonication and centrifuged at 105,000 *g* for 30 min. The supernatants containing the cytosolic proteins were kept at −20°C until used for Western-blot analysis of the 3 isoforms of GST (π, α and μ.).

The pellets were re-suspended in lysis buffer containing 1% Triton X-100 by sonication and the membrane suspensions were centrifuged again at 105,000 *g* for 30 min. The supernatants containing the membrane proteins were kept at −20°C until used for Western-blot analysis of c-erbB-2 gene product.

Samples were normalized for protein content. Cell lysates were separated by SDS-PAGE. Resolved proteins were electrotransferred to nitro-cellulose and probed with polyclonal anti-human GST π, α, and μ (Bioprep, Dublin, Ireland) or polyclonal anti-human c-erbB-2 antibodies (Triton, Alameda, CA). The secondary antibody was conjugated to peroxidase (Bio-Rad, Ivry Sur Seine, France). Protein bands were visualized by the colorimetric method using the substrate 4-chloronaphtol.

Total GSH concentration

The sum of the reduced and oxidized forms of glutathione were determined in cytosolic fractions from lysed cells using the method of Akerboom and Sies (1981). In this assay, the sum of the reduced and oxidized forms of glutathione are determined using a kinetic assay in which catalytic amounts of reduced or oxidized glutathione and glutathione reductase bring about the continuous reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by NADPH. The reaction rate is proportional to the concentration of GSH below 2 μM. The formation of 5-thio-2-nitrobenzoate is followed spectrophotometrically at 412 nm. The results were expressed as nmol per mg of protein, and compared with corresponding control data by the Student *t*-test.

CDNB-glutathione S-transferase (GST) activity

GST was assayed as described by Habig *et al.* (1974), using CDNB as substrate. Formation of 1-chloro-2,4-dinitrobenzene-GSH conjugate by cytosols was measured continuously in a spectrophotometer at 340 nm. The results were expressed in nmol of CDNB conjugated per min per mg of cytosolic protein, and compared with corresponding control data by the Student *t*-test.

Total RNA extraction and Northern blot analysis of metallothionein mRNA

2008 and 2008/C13* cell lines in sub-confluence phase were treated with their respective IC_{50} of IFNγ. Total RNA was isolated from cells, after 4-, 8- and 24-hr exposure to IFNγ, by acid-guanidinium-thiocyanate-phenol-chloroform extraction. Total RNA (20 μg) were electrophoresed on 1.2% agarose gels in the presence of formaldehyde (6.5%), transferred to Hybond nylon membranes (Amersham) and fixed by UV light.

Membranes were pre-hybridized at 42°C for 8 hr in a solution composed of 50% formamide, 6× SSPE, 5× Denhardt's solution, 1% SDS and 200 μg/ml denatured salmon sperm DNA. Hybridization with DNA probe for metallothionein (MT) was carried out overnight at 42°C. The probe used is hMT-II, which encodes human metallothionein II (Karin and Richards, 1982), obtained from the ATCC (Rockville, MD). The radioactivity of [α-^{32}P]dCTP-labeled hMT-II DNA probe was 2.10^6 cpm/ml. After hybridization, the membranes were washed twice for 15 min in a solution of 2× SSC and 0.1% SDS at 42°C and twice for 30 min in a solution of 0.5× SSC and 0.1% SDS at 55°C. Autoradiograms of hybridized membranes were obtained using Hyperfilms-MP (Amersham) exposed at −80°C.

Densitometry

Levels of protein or mRNA were semiquantitated using a Kodak DCS 200 densitometer.

DNA adduct formation and repair

Cells were grown in Falcon cell-culture flasks (Nunc) (175 cm^2) to 50 to 80% confluence, and then labelled with ^{3}H-thymidine (0.2 μCi/ml) for 24 hr. Cells were washed twice with PBS, trypsinized, dispersed and plated with fresh medium onto plastic tissue-culture dishes (60 mm^2) at a density of 6.10^6 cells/dish. Twenty-four hours later, cells were exposed to 200 μM (60 μg/ml) cisplatin for 1 hr, with or without IFNγ concentrations of 1,200 and 600 U/ml for 2008 and 2008/C13* cells respectively, as defined by the fixed ratio of concentrations of the 2 drugs used for median-effect analysis in our earlier work (Nehmé *et al.*, 1994). Cells were then harvested at the following time points: 0, 6 and 24 hr later. Total cellular DNA was extracted with buffered phenol: chloroform (1:1) after digestion with 100 μg/ml proteinase K and 0.5% SDS in digestion buffer (10 mM Tris-HCL, 0.1 mM EDTA and 20 μg/ml RNase A, pH 8.0) for 4 hr at 50°C and ethanol precipitation. DNA concentration was determined by optical density at 260 nm. ^{3}H-thymidine content was assessed by liquid-scintillation counting, and total platinum content was assessed by atomic absorption spectrophotometry. Platinum content in cellular DNA at each time point was corrected for DNA replication based on the original ^{3}H-thymidine content of cellular DNA at time 0 hr.

RESULTS

Effect of IFNγ on CDDP accumulation

The human ovarian carcinoma 2008 and 2008/C13* cell lines were incubated with their respective IC_{20} (0.4 and 4 μg/ml) and IC_{50} (1 and 9 μg/ml) of CDDP (concentrations causing 20% and 50% inhibition of cell growth respectively) in the presence or absence of corresponding concentrations of IFNγ, as described in "Material and Methods". Figure 1 shows that IFNγ had no effect on the accumulation of cisplatin for 1 hr.

Effect of IFNγ on GSH content and GST activity

Tissue-culture conditions have been shown to influence significantly the cellular GSH level in human ovarian-cancer cell lines (Batist *et al.*, 1986). For this reason, the cellular GSH level and the GST activity have been serially determined in a human ovarian-cancer cell line (2008) and its 10-fold-CDDP-resistant subline (2008/C13*), with or without treatment with IFNγ for 0 to 72 hr. Serial GSH determinations for the 2 cell lines are shown in Table I. As reported by Batist *et al.* (1986), total GSH levels were maximal in both cell lines, with or without treatment with IFNγ, early after sub-culture (4, 12 and 24 hr) and then declined. At 12 hr, in control cells, the GSH levels were 54 ± 9 and 116 ± 14 nmole/mg protein in 2008 and 2008/C13* cells respectively. GSH level was significantly reduced in 2008 cells (39 ± 6 nmole/mg protein, $p < 0.05$) and in 2008/C13* cells (56 ± 2 nmole/mg protein, $p < 0.05$) 12 hr after treatment with IFNγ. Moreover, the GSH level was also significantly decreased in 2008/C13* cells treated with IFNγ for 4 hr (49 ± 8 nmole/mg protein, $p < 0.05$), as compared with the control cells. However, GSH levels returned to the normal ranges determined serially between 48 and 72 hr after adding IFNγ.

By contrast, we did not observe significant fluctuations in the GST activity over time (Table II). IFNγ significantly decreased total GST activity both in 2008 and in 2008/C13* cell lines after 72 hr of treatment (Table II). Total GST activities were 1170 ± 160 and 1350 ± 540 nmole CDNB/min/mg protein in 2008 and 2008/C13* cells respectively. GST activity was reduced in 2008 cells (617 ± 116 nmole CDNB/min/mg pro-

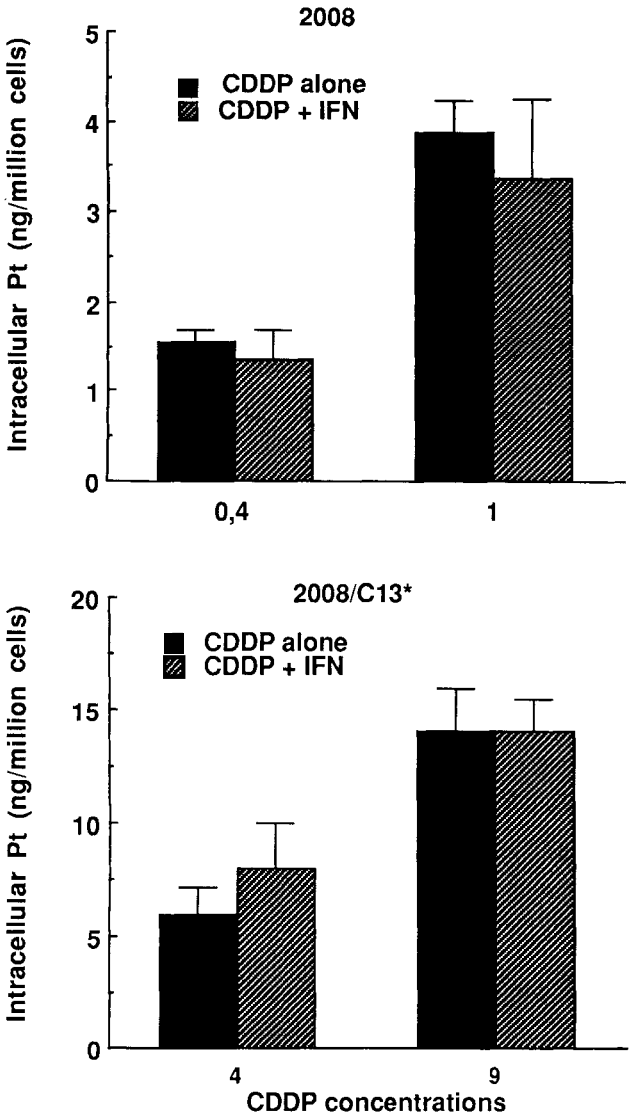

FIGURE 1 – Effect of IFNγ on platinum accumulation in 2008 and 2008/C13* cell lines. Cells were treated with CDDP IC_{20} and IC_{50} for 1 hr with or without corresponding concentrations of IFNγ, as described in "Material and Methods". Each histogram represents the mean of 3 experiments. Bars, SD.

tein, $p < 0.01$) and in 2008/C13* cells (710 ± 200 nmole CDNB/min/mg protein, $p < 0.05$) 72 hr after treatment with IFNγ. It is important to note that GST activity was also decreased in the 2008 cell line treated with IFNγ for 48 hr (788.5 ± 46 nmole CDNB/min/mg protein, $p < 0.05$), as compared with the control cells.

To assess the glutathione transferase sub-units affected by interferon treatment, expression of the 3 major cytosolic classes of glutathione-S-transferase (GST π, α and μ) was examined by Western blotting of material obtained from lysed cells. There was no detectable level of α and μ sub-units in the 2008 or the 2008C13* cell lines (data not shown), whereas the π gene product was detected in these 2 cell lines. The absence of a reference signal for GSTα and GSTμ precluded that the decrease in GST activity might be due to a decrease in the π isoform.

The effect of IFNγ on the relative level of the GSTπ sub-unit in the 2 ovarian-cancer cell lines is shown in Figure 2. Expression of the π gene product was decreased in 2008 cell

TABLE I – EFFECT OF IFNγ ON GSH LEVELS IN 2008 AND 2008/C13* CELL LINES

Time (hr)	GSH nmole/mg protein			
	2008		2008/C13*	
	−IFNγ	+IFNγ	−IFNγ	+IFNγ
4	48 ± 6	43 ± 2	84 ± 9	49 ± 8*
12	54 ± 9	39 ± 6*	116 ± 14	56 ± 1*
24	63 ± 4	46 ± 12	107 ± 22	65 ± 22
48	30 ± 5	26 ± 4	97 ± 2	55 ± 5
72	26 ± 5	35 ± 4	66 ± 4	58 ± 3

Cells were treated with IFNγ IC_{50} for 0 to 72 hr and harvested for up to 72 hr (4, 12, 24, 48 and 72 hr). Total GSH levels were measured at each time point in the 2 cell lines with or without treatment with IFNγ. The results were expressed as nmole per mg of protein. Each point represents the mean of 3 experiments.– *Significantly different from control at $p < 0.05$.

TABLE II – EFFECT OF IFNγ ON GST ACTIVITY IN 2008 AND 2008/C13* CELL LINES

Time (hr)	GST activity nmole CDNB/mg protein			
	2008		2008/C13*	
	−IFNγ	+IFNγ	−IFNγ	+IFNγ
4	935 ± 115	876 ± 144	1777 ± 143	1724 ± 194
12	1115 ± 132	1150 ± 134	1719 ± 149	1710 ± 128
24	962 ± 175	1049 ± 107	1623 ± 495	1763 ± 355
48	1219 ± 25	788.5 ± 46*	1294 ± 126	1215 ± 64
72	1170 ± 160	617 ± 116**	1350 ± 540	710 ± 200*

Cells were treated with IFNγ IC_{50} for 0 to 72 hr and harvested for up to 72 hr (4, 12, 24, 48 and 72 hr). GST activities were measured at each time point in the 2 cell lines with or without treatment with IFNγ. The results were expressed as nmole CDNB conjugated per mg of protein. Each point represents the mean of 3 experiments, * and ** Significantly different from control at $p <$ 0.05 and 0.01 respectively.

line by approximately 50% after treatment with IC_{20} and IC_{50} of IFNγ for 3 days, as determined by densitometric tracings. However, in the CDDP-resistant cell line 2008/C13*, this decrease in GSTπ expression was lower (25%) and observed only after treatment with IC_{50} of IFNγ.

Effect of IFNγ on cellular metallothionein mRNA

A Northern blot analysis was undertaken to investigate the effect of IFNγ on the metallothionein gene expression in 2008 and 2008/C13* cell lines. Figure 3 shows the mRNA levels for $hMTII_A$. The GAPDH probe (Fort *et al.*, 1985) was used to confirm the equivalent lane loading of total RNA. IFNγ increased the level of metallothionein II_A both in the 2008 and in the 2008/C13* cell lines. mRNA $MTII_A$ reached a maximum about 8 hr after IFNγ treatment. The mRNA level fell rapidly between 8 and 24 hr after adding IFNγ.

Effect of IFNγ on DNA adduct formation and repair

Table III shows the data obtained for the formation and removal of total cisplatin lesions in genomic DNA of the parental and cisplatin-resistant cell lines treated with 200 μM cisplatin for 1 hr with or without corresponding concentrations of IFNγ, as described in "Material and Methods". The IFNγ produced no significant effect on adduct formation measured immediately after the end of the cisplatin exposure, in the 2008 and the 2008/C13* cell lines. The rate of adduct repair was estimated from the rate of loss of platinum from the DNA corrected for new DNA synthesis. Table III shows that over a 24-hr period following 1-hr cisplatin exposure (200 μM), the cisplatin-resistant 2008/C13* cell line removed approximately 38% of the total adduct present at the end of the cisplatin exposure; IFNγ had no effect on adduct repair.

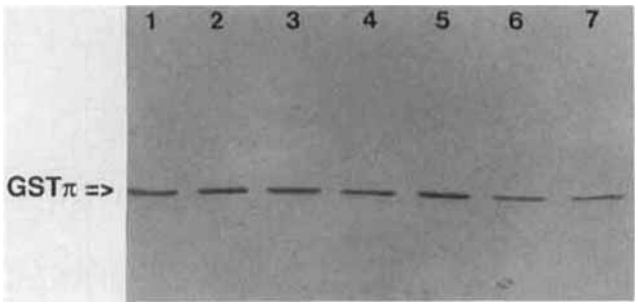

FIGURE 2 – Immunoblot analysis of the GSTπ sub-unit in 2008 and 2008/C13* cell lines after treatment with IFNγ IC_{20} and IC_{50} for 72 hr. Equal amounts of protein (20 μg) were separated through SDS/15% polyacrylamide gel before electroblot transfer to nitrocellulose filters. The GSTπ protein was detected using the rabbit polyclonal anti-human GSTπ as described in "Material and Methods". Lane 1, control: human breast tumor; lane 2, 2008/C13* (untreated cells); lane 3, 2008/C13* treated with IFNγ IC_{20} (10 U/ml); lane 4, 2008/C13* treated with IFNγ IC_{50} (80 U/ml); lane 5, 2008 (untreated cells); lane 6, 2008 treated with IFNγ IC_{20} (7 U/ml); lane 7, 2008 treated with IFNγ IC_{50} (20 U/ml).

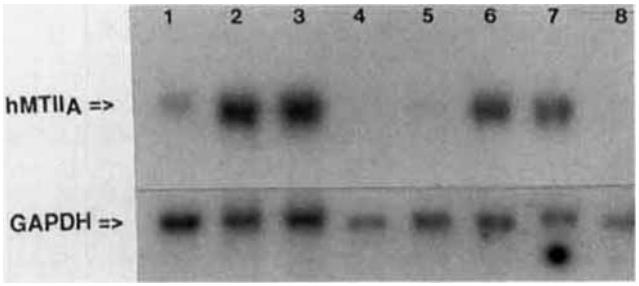

FIGURE 3 – Northern-blot analysis of the $hMTII_A$ mRNA level in 2008 and 2008/C13* cell lines after treatment with IFNγ IC_{50}. Total RNA (20 μg) was electrophoresed, blotted and hybridized as described in "Material and Methods". All hybridizations were performed on the same filter, and hybridization with GAPDH was used to show the equivalent loading of the total RNA. Lane 1, 2008/C13* (untreated cells); lane 2, 2008/C13* treated with IFNγ for 4 hr; lane 3, 2008/C13* treated with IFNγ for 8 hr; lane 4, 2008/C13* treated with IFNγ for 24 hr; lane 5, 2008 (untreated cells); lane 6, 2008 treated with IFNγ for 4 hr; lane 7, 2008 treated with IFNγ for 8 hr; lane 8, 2008 treated with IFNγ for 24 hr.

*Effect of IFNγ on c-*erb*B-2 proto-oncogene expression*

The effect of IFNγ on the expression of c-*erb*B-2 was demonstrated by immunoblotting the membrane fractions obtained from lysed cells. Reduced levels of the M_r 185.000 protein were detected both in the 2008 and in the 2008/C13* cell lines (Fig. 4). The reduction of c-*erb*B-2 expression was dependent on the concentrations of IFNγ. In 2008 cells, densitometric analysis revealed a decrease in expression of c-erbB-2 protein by approximately 50% after 3 days of treatment with IFNγ IC_{50}, whereas in 2008/C13* cells there was no detectable level of c-erbB-2 protein after adding IFNγ IC_{50}.

DISCUSSION

In this study, we have investigated the mechanism by which IFNγ synergizes the cytotoxicity of CDDP as described (Nehmé *et al.,* 1994). The mechanism of synergy between these agents may involve modulation of the cellular accumulation of CDDP, as demonstrated for different drugs such as IL-1α (Benchekroun *et al.,* 1993), dipyridamole (Jekunen *et al.,* 1992), forskolin

TABLE III – EFFECT OF IFNγ ON THE FORMATION AND REMOVAL OF CISPLATIN ADDUCTS FROM THE OVERALL GENOME IN CISPLATIN-SENSITIVE CELL LINE 2008 AND ITS 10-FOLD-CISPLATIN-RESISTANT SUB-LINE 2008/C13*

IFNγ	Repair time (hr)	2008 Sensitive cells		2008/C13* Resistant cells	
		Pt-adducts pg/μg DNA	Repair (%)	Pt-adducts pg/μg DNA	Repair (%)
−	0	104 ± 12		32.5 ± 2.5	
+	0	116 ± 16.6		31 ± 3	
−	6	115 ± 12.6	0	36 ± 2	0
+	6	129 ± 14	0	39 ± 0.8	0
−	24	N.E.	N.E.	11 ± 1.2	38
+	24	N.E.	N.E.	10 ± 2.3	40

Cells were treated with CDDP (200 μM) for 1 h with or without corresponding concentrations of IFNγ as described in "Material and Methods". Each point represents the mean of 3 experiments. NE, not evaluable.

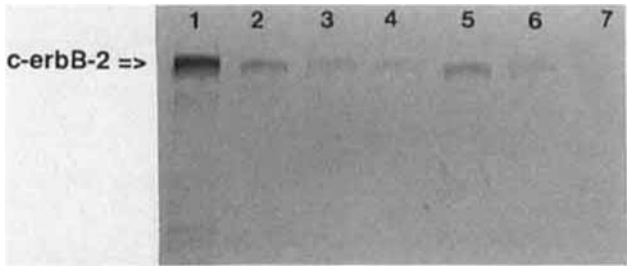

FIGURE 4 – Detection of c-erbB-2 protein by Western blotting. The 2 ovarian-carcinoma cell lines, 2008 and 2008/C13*, were treated with IFNγ IC_{20} and IC_{50} for 72 hr. Protein extract (50 μg) of each indicated cell line were separated on SDS/6% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The c-erbB-2 protein was detected with the polyclonal rabbit anti-human c-*erb*B-2 as described in "Material and Methods". Lane 1, control: human breast cancer cell line SKBR-3; lane 2, 2008 (untreated cells); lane 3, 2008 treated with IFNγ IC_{20} (7 U/ml); lane 4, 2008 treated with IFNγ IC_{50} (20 U/ml); lane 5, 2008/C13* (untreated cells); lane 6, 2008/C13* treated with IFNγ IC_{20} (10 U/ml); lane 7, 2008/C13* treated with IFNγ IC_{50} (80 U/ml).

(Mann *et al.,* 1991) or amphotericin B (Morikage *et al.,* 1993). It was not the case for IFNγ, which has no effect on cellular platinum uptake. The intracellular levels of GSH and total GST enzyme activity have been reported to influence sensitivity to CDDP (Chen *et al.,* 1989). The present studies indicate that, for the 2008 and the 2008/C13* cell lines, exposure of the cells to the concentrations of IFNγ used in the synergy studies caused a significant time-dependent decrease of cellular GSH. Interestingly, it has been reported that prolonged GSH depletion can sensitize human ovarian-carcinoma cells to CDDP cytotoxicity (Andrews *et al.,* 1988*a*). All these observations suggest that, for 2008 and 2008/C13* cell lines, the synergistic interaction between CDDP and IFNγ may involve a modulation of cellular GSH by IFNγ. The present studies also showed a significant time-dependent decrease in total GST activity in 2008 and 2008/C13* cell lines after treatment with IFNγ. While mechanisms of regulation of GST activity by IFNγ in ovarian-cancer cell lines are not clear, decreased levels of this drug-metabolizing enzyme activity may be one of reasons of the observed synergy between IFNγ and CDDP. IFNγ has been reported to cause significant changes in the levels of the cytosolic hepatic glutathione transferases in mice; thus, the GST enzyme represents a potentially important interferon-modulated gene product (Adams *et al.,* 1987). Western-blot analysis of cytosolic protein from lysed cells showed undetectable levels of the gene product for the GST classes α and μ,

whereas the GST π gene product was expressed in both the 2008 and the 2008/C13* cell lines. These results agree with those of Murphy *et al.* (1992), who demonstrated that the expression of basic and neutral GST iso-enzymes was weak or absent in ovarian tumors.

IFNγ caused a significant decrease of the level of GSTπ isozyme in 2008 and 2008/C13* cell lines. The changes in GSTπ protein level following IFNγ treatment were in some degree quantitatively confirmed by changes in CDNB substrate metabolism, as discussed above. These data indicate that the reduced amount of GST π-sub-unit protein after treatment with IFNγ might represent a mechanism by which IFNγ inhibits total GST activity.

Although metallothionein II_A mRNA and not protein levels were measured in this study, the fact that IFNγ causes a substantial increase in message level makes it unlikely that it would have decreased the metallothionein II_A protein. Such an induction has been reported with 12-O-tetradecanoyl-phorbol-13-acetate in the same cell line (Isonishi *et al.*, 1994). This induction in metallothionein II_A content was the opposite of what might be expected if IFN-induced sensitization was linking through reduction of metallothionein II_A content.

It is generally considered that DNA is the most important cytotoxic target for CDDP. DNA-intra-strand cross-links, DNA-interstrand cross-links and DNA-protein cross-links are induced by CDDP, but there is no agreement as to which of these lesions is responsible for the cytotoxicity of CDDP (Kelland, 1993). Although IFNγ did not affect the cellular uptake of CDDP during the 1-hr CDDP exposure, it must alter processes that influence toxicity during the period after removal of the cells: DNA-interstrand-adduct formation continues for several hours after CDDP removal, and repair of inter- and intra-strand adduct continues for 24 hr or more (Micetich *et al.*, 1983). It is important to note, however, that a 1-hr treatment with CDDP is optimal for the generation of intra-strand adducts, whereas the optimal formation of inter-strand cross-links requires 5-hr treatment with CDDP (Jones *et al.*, 1991). IFNγ caused no increase in adduct formation and no change in the rate of adduct removal over the 24 hr following 1-hr exposure to CDDP. It is important to note, however, that the technique used quantified only the extent of DNA platination and the excision of total platinum in DNA. It is possible that IFNγ may alter the formation and repair of inter-strand cross-links, or intra-strand adducts in the overall genome, and/or in the specific genomic regions of DNA (Link *et al.*, 1991; Zhen *et al.*, 1992).

The altered expression of proto-oncogenes has been reported to play a role in the acquisition of cisplatin resistance (Andrews and Howell, 1990; Kelland, 1993; Timmer-Bosscha *et al.*, 1992). The experiments presented in this study provide evidence that IFNγ inhibits the expression of the c-*erb*B-2 oncogene both in 2008 and in 2008/C13* cell lines. Our findings are in agreement with earlier reports that IFNγ reduces the amount of c-erbB-2 protein in ovarian-carcinoma cells and that IFNγ exerts its effects by reducing the amount of c-erbB-2 mRNA (Marth *et al.*, 1990). The normal function of the c-*erb*B-2 oncogene is not fully understood, but it is known that the c-erbB-2 gene product has the structural features and many of the functional properties of sub-class-I-growth-factor receptors. Moreover, it has been demonstrated that a monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of CDDP against human breast and ovarian tumor cell lines both *in vitro* and *in vivo* (Hancock *et al.*, 1991). Considering these results, the down-regulation of c-*erb*B-2 appears to be one of the possible mechanisms involved in the synergistic interaction between CDDP and IFNγ.

The IFNγ-mediated modulation of GSH content, GST π activity and c-*erb*B-2 oncogene expression may be a central point in the regulation of CDDP cytotoxicity by IFNγ in ovarian-cancer cell lines. However, the network of interaction between these 2 drugs is and may involve additional mechanisms.

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