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Use of a monoclonal antibody to detect DNA damage caused by the anticancer drug *cis*-diamminedichloroplatinum (II) in vivo and in vitro

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Abstract A monoclonal antibody, MAb62-5, was prepared and used to detect DNA damage due to the anticancer drug *cis*-diamminedichloroplatinum (II) (or cisplatin). ELISA competition indicated that the binding of MAb62-5 to cisplatin–DNA was competitively inhibited (50% control) by 210 nM of cisplatin bound to DNA, cisplatin/nucleotide (D/N) = 0.2. Using a DNA mobility shift assay, MAb62-5 binding activity was inhibited by 50% by ~50-fold molar excess of cisplatin–DNA adducts (D/N = 0.08), whereas there was less than 5% inhibition by UV-DNA adducts or mock-treated DNA. In addition, MAb62-5 showed a similar affinity to the cisplatin–DNA adducts as compared to an endogenous cisplatin-damaged DNA recognition protein. Using ELISA with this antibody, we have demonstrated a 2-fold enhancement in excision repair of cisplatin-DNA adducts in resistant HeLa cells. This is supported by the measurement of repair-associated DNA strand breaks using alkaline elution and host cell reactivation of transfected plasmid DNA carrying cisplatin damage. These findings also provide a possible explanation for the complexicity of immunoassay in cells.

Key words: Cisplatin; DNA repair; Damage-recognition protein; ELISA

1. Introduction

cis-Diamminedichloroplatinum (II), or cisplatin, is a widely used chemotherapeutic agent [1,2]. Occasionally, resistant cells arise during the course of treatment, hindering cancer therapy. It has been demonstrated that cisplatin is an effective crosslinking agent which generates various forms of cisplatin–DNA adducts [3]. Although the lethal target of cisplatin and the mechanism for the resistant phenotype are not clear, the interaction of cisplatin with DNA has been implicated as the major cytotoxic action of the drug [4]. A number of important questions regarding the failure of the treatment in clinics could be answered directly if the extent of these DNA interactions could be reliably measured from small numbers of cells removed from patients. In the past, the majority of studies dealing with the quantitation of cisplatin–DNA adducts in cells involved the isolation of genomic DNA from treated cells, and then measurement by atomic absorption, alkaline elution, or sedimentation. We and others have recently established an indirect method, i.e. host cell reactivation of damaged plasmid DNA, to detect DNA repair, in which cisplatin–DNA adducts are prepared in vitro prior to introduction into cells without modifying the cellular repair machinery [5,6]. However, any one of the afore-mentioned methods is not always applicable to all situations. Thus, the development of a simple and sensitive assay is required for the detection of a low level of cisplatin–DNA adducts prior to the emergence of resistant phenotypes in cancer therapy.

Most cisplatin-resistant cells are also resistant to alkylating agents and cadmium, or exhibit an alteration in their level of free radical scavengers like glutathione or metallothionein [3,7], suggesting that cisplatin resistance may be related to radical-mediated DNA damage and repair. Using a cell-free repair system, independent investigations have demonstrated that cell extracts can carry out repair synthesis in DNA damaged by UV, psoralens and platinating agents [8–13], whereas extracts from some xeroderma pigmentosum (XP) cell lines are incapable of repairing damaged plasmid DNA [8,14]. Recently, damaged-DNA binding proteins which have been identified from calf thymus, can complement DNA repair of XPA cell extracts [15], whereas cell extracts from XP group A or E cells display a reduced recognition of UV-damaged DNA [16–18]. These results suggest that the capability of cells in eliminating damaged-DNA, largely rate-limited at the early stage of excision repair, plays an important role in the sensitivity or resistance of cells to genotoxic agents. We have previously established a cisplatin-resistant HeLa cell line [6], which is phenotypically cross-resistant to UV irradiation and overproduces damaged-DNA recognition proteins [9,20]. Using a cell-free system [8], we have demonstrated an improved recognition and incision of UV–DNA adducts as a potential indicator of UV resistance [21]. In this study, we described the production and initial characterization of a monoclonal antibody specific for cisplatin–DNA adducts. This highly sensitive and simple assay was compared with other methods and was used to investigate the accumulation of cisplatin–DNA adducts in the resistant as well as the parental HeLa cells. The data indicates that reduced adduct frequency and enhanced DNA excision repair, being rate-limited in the early step, are potential mechanisms of cisplatin resistance in human cells. The findings from the DNA mobility shift analysis also suggest that the monoclonal antibody preferentially recognizes cisplatin–DNA adducts, the same target of an endogeneous nuclear protein of cells.

2. Materials and methods

2.1. Media, cell cultures, and cytotoxicity

Human cervix carcinoma HeLa cells and cisplatin-resistant HeLa-CPR variants [6] were maintained in a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin, and were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. 1 μM cisplatin was added to the medium to maintain the resistant

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Abbreviations: cisplatin, *cis*-diamminedichloroplatinum (II); CDRP, cisplatin-damaged DNA recognition protein; ELISA, enzyme-linked immunoassay; UV, ultraviolet radiation.

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phenotype. Prior to experiments, the resistant cells were grown continuously in media without cisplatin for three population doublings. The sensitivity of cells to cisplatin was determined from clonogenicity of cells 2 weeks following treatment. The fold resistance of cells was calculated by the ratio of IC_{50}, cisplatin concentration inhibiting 50% of cell proliferation, of the resistant cells vs. that of the parental HeLa cells. All the culture media and antibiotics were purchased from Gibco, Gaithersburg, MD; other chemicals were from Sigma, St. Louis, MO, unless otherwise indicated.

2.2. Antibodies and enzyme-linked immunosorbent assay (ELISA)

Monoclonal antibody MAb 62-5, which preferentially recognizes cisplatin–DNA adducts, was prepared according to the method of Sundquist et al. [22]. Briefly, calf thymus DNA (Serva) was platinated to bound cisplatin D/N = 0.21 as measured by atomic absorption spectroscopy [23]. Exactly 0.15 mg of platinated DNA was complexed to methylated BSA in 0.9% NaCl, and prepared as an immunogen to immunize Balb/c mice. Before each cell fusion during hybrodoma production, blood samples were removed from the mouse tails and estimated for antibody activity. For ELISA of antibody activity, polystyrene flat-bottomed 96-well microtiter plates (diameter 3.4 mm, Corning) were coated with 3 μg cisplatin–DNA (calf thymus DNA) (D/N = 0.2) in 100 μl PBS and air-dried overnight at room temperature. The non-specific binding of cells was blocked by adding 1% normal goat serum (NGS) in PBS at 37°C for 60 min. After removal of NGS with PBS containing 0.05% Tween, the plate was incubated in 100 μl diluted MAb 62-5 (1:25) in the absence or presence of competitors for 30 min at 37°C. Secondary antibody (50 μl), peroxidase-conjugated goat anti-mouse immunoglobulins (Dako A/S, Copenhagen, Denmark), was added and incubated at 37°C for 30 min. Freshly prepared 1 mM ABTS (2,2'-azino-bis(3-ethy(benzthiazoline-6-sulfonic acid) in ABTS buffer (0.1 M citrate, 0.2 M disodium phosphate buffer, pH 4.2–4.8, 0.01% H_2O_2) was then added for 30 min and the absorbance at wavelength 405 nm was read with a Biotek microtiter plate reader as previously described [22,24,25].

2.3. Alkaline elution and measurement of single-stranded DNA breaks

Single-stranded DNA breaks were estimated using alkaline elution as previously described [26]. Cellular DNA was labeled with 0.01 μCi/ml [2-^{14}C]thymidine (51.4 Ci/mmol) or [*methyl*-^{3}H]thymidine (25 Ci/mmol). Following treatment of cells with cisplatin, the ^{14}C-labeled cells were incubated for 0–20 min in medium containing 10 mM hydroxyurea (HU) and 0.1 mM 1-β-D-arabinofuranosyl cytosine (ara-C). ^{3}H-Labeled control cells were also incubated with the inhibitors. Cells were harvested in ice-cold PBS containing 0.2 mg/ml Na_2EDTA. ^{14}C-Labeled cells were mixed with ^{3}H-labeled control cells, and subjected to alkaline elution. The elution conditions and calculation of DNA strand breaks were performed as described by Rosenstein and Ducore [27].

2.4. DNA transfection and CAT assay

20 μg each of pRSVcat and pSVβ (Clontech Laboratories Inc.) plasmid DNA was co-transfected into cells using the electroporation technique [28]. pRSVcat was treated with cisplatin in vitro to generate various extents of cisplatin–DNA adducts prior to being introduced into cells as described before [19]. 1 ml of the cell suspension, in HEPES buffer, was added to a sterile cuvette containing pRSVcat and pSVβ plasmid, gently mixed, and subjected to electroporation by GenePulser (Bio-Rad) with 1000 μF capacity and 200 V. Following 40 h incubation in normal medium, cells were harvested into 1 ml PBS from which 200 μg and 400 μg of cell extracts was prepared for CAT and β-galactosidase activity assays [29], respectively. The CAT assay reaction was incubated at 37°C for 1 h, followed by development on a silica thin-layer chromatography (TLC) plate (Macherey-Nagel, Germany). After autoradiography, density on the X-ray film corresponding to the modified chloramphenicol or not was quantitated through a scanning densitometer (Hoefer GS300). The average of three scans of each chloramphenicol corresponding spot was taken. CAT activity was calculated as per cent of chloramphenicol substrate converted into acetylated derivatives. After being normalized to β-galactosidase activity, relative CAT activity was determined by setting untreated cells as 100%.

2.5. DNA probes, nuclear extracts, and DNA mobility shift assay

The *Hin*dIII–*Eco*RI f103 fragment prepared from pGC14 was treated with cisplatin or UV as previously described [18]. Briefly, *Hin*dIII and *Eco*RI generated DNA fragments were labeled with [^{32}P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL) to 3×10^4 cpm/ng DNA using Klenow DNA polymerase and purified by column chromatography using standard methods [29]. The DNA at a concentration of 100 μg/ml was platinated according to [51] or UV irradiated as described before [26]. The extent of platination of f103 was estimated by atomic absorption spectroscopy as described [23]. Alternatively, f103 DNA was irradiated with germicidal lamps via a VL-100C UV irradiation unit (Vilbert Lourmat, France) at a fluence rate 25 J/m^2/s. The fluence rate was measured by a VLX-254 radiometer (Vilbert Lourmat, France). Nuclear extracts were prepared according to Dignam et al. [31]. The protein concentration was measured via the Bradford assay using the Bio-Rad dye reagent [32], and visualized by SDS-PAGE [33]. Protein–DNA binding was performed according to Hannighausen and Lubon [34] using antibody MAb62-5 (1:25 dilution) or nuclear extracts in a 15 μl of buffer containing 12% glycerol 12 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 300 μg/ml BSA and 2 μg poly(dI-dC) at 25°C for 30 min as described. The reaction mixtures were then subjected to 5% polyacrylamide gel electrophoresis under low ionic strength (6.7 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) at 30°C and 15 mA constant current. The resolved gel was dried and exposed to Kodak XAR-5 X-ray film with an intensifying screen at −70°C. The intensity of the shifted DNA bands was determined from the average of three scans by scanning densitometry.

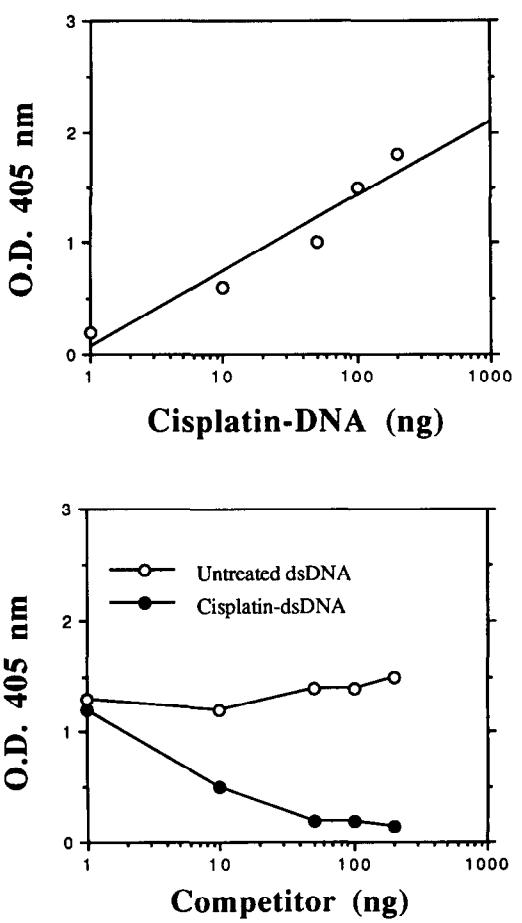

Fig. 1. Specificity of MAb62-5 as assayed by ELISA. (Upper panel) Dose–response of MAb62-5 binding. The line was calculated by linear regression as $y = 0.067345 + 0.68047 \cdot \mathrm{LOG}(x)$; $R^2 = 0.928$, where R is the correlation coefficient. (Lower panel) Competition of MAb62-5 binding. The indicated amounts of competitors, untreated or cisplatin-treated double-stranded (ds) DNA adducts (D/N = 0.2), were added to the binding reaction.

3. Results

3.1. Specificity of the monoclonal antibody

The specificity of MAb62-5 (1:25 dilution) was characterized in vitro by ELISA (Fig. 1). Exactly 0, 10, 50, 100 or 200 ng of platinated DNA (D/N = 0.2) was used to generate a dose–response curve (Upper Panel). The $OD_{405\,nm}$ increased with the amount of cisplatin–DNA, giving a regression line with a slope of 0.68047. For competition analysis, a 96-well plate was coated with platinated DNA (D/N = 0.2). Exactly 0, 10, 50, 100 or 200 ng of untreated or platinated DNA was used as competitor. The competition curves were determined by the $OD_{405\,nm}$ vs. the amount of competitor (Fig. 1, lower panel). Apparently, control DNA (open symbols) did not affect MAb binding. In contrast, more than 50% of the binding was inhibited by 10 ng or more cisplatin–DNA (filled symbols). For comparison, competitors other than cisplatin-treated DNA were also analyzed. The IC_{50} values derived from the competition curves were determined (Table 1). Cisplatin-treated ssDNA or poly(dG)·poly(dC) with indicated D/N, respectively, displayed 17- and 100-fold less effective inhibition than the cisplatin-treated dsDNA. About 300 fold of transplatin-treated dsDNA or ssDNA was needed to inhibit the same level of immunoreactivity. Additionally, the specificity of MAb62-5 in a 1:50 dilution generated similar results.

Cellular specificity of MAb62-5 was also demonstrated by competitive ELISA (Fig. 2). DNA from cells treated with 0, 15, 50 or 150 μM cisplatin was isolated and the relative $OD_{405\,nm}$ was determined. In all these assays in which 50% binding was inhibited by ~10 fold competitor the competition patterns were similar or the same. As shown, more than 90% binding was inhibited by 100 fold competitor. Compared with untreated cells, a ~10^2, 10^6 and 10^7 fold difference in the amount of competitor is required to inhibit 50% binding in cells treated with 15, 50 and 150 μM cisplatin, respectively. The level of DNA adducts determined by atomic absorption spectrophotometry in 15 μM cisplatin-treated HeLa and a derivative cell line is ~890 and 340 nmol cisplatin per g of DNA, respectively [35]. Using ELISA, induced DNA adducts were also detected in 5 μM cisplatin-treated cells (data not shown). The results indicate that MAb62-5 preferentially interacts with cisplatin–DNA adducts in vitro and in vivo.

3.2. Detection of MAb62-5 binding activity by DNA mobility shift assay

MAb62-5 binding activity was detected by DNA mobility shift assay (Fig. 3). Under the standard DNA-binding conditions with MAb62-5 at 1:25 dilution, in the absence (lane 2) or presence of 10-fold (lanes 4, 6 and 8) or 100-fold (lanes 5, 7 and 9) of indicated competitors, a binding activity was detected (indicated with an arrowhead). MAb62-5 binding activity was inhibited by ~30% by a 100-fold f103-pt, whereas greater than 90% binding was inhibited by a 1000-fold f103-pt. In contrast, the binding remained intact with either 100 or 1000 fold f103 or f103-uv. As also shown, an additional binding band was detected by undiluted MAb62-5 (lane 3). The latter was probably due to interaction of a platinated DNA with more than one antibody molecule since D/N = 0.08 corresponds to ~10 cisplatin per f103. Reaction with probe alone showed only free probe (indicated with a star) and non-specific binding. Thus, the immunoreactivity of MAb62-5 could be detected in a DNA mobility shift assay.

Table 1
Immunoreactivity of MAb62-5 measured by ELISA

Competitor[a]	D/N[b]	IC_{50} (ng)[c]
Cisplatin–dsDNA	0.21	7
Cisplatin–ssDNA	0.14	120
Transplatin–dsDNA	0.42	2500
Transplatin–ssDNA	0.49	2000

[a]ssDNA, single-stranded DNA prepared by boiling dsDNA for 10 min and rapid cooling at 4°C.
[b]D/N, competitor DNA at bound platinum/nucleotide ratio as determined by atomic absorption spectroscopy [23].
[c]Amounts of platinated DNA which caused 50% inhibition of the binding.

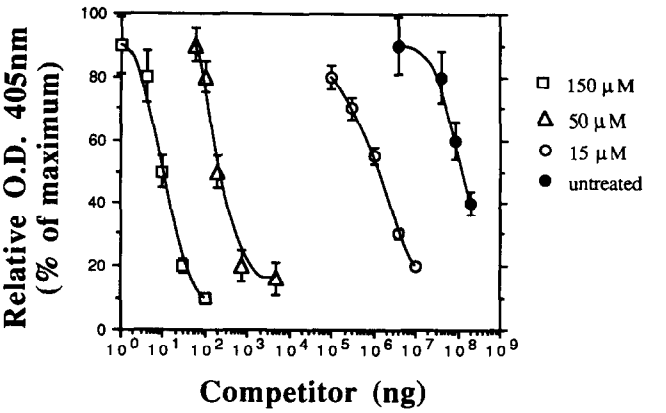

Fig. 2. Competitive ELISA for DNA extracted from cells that had been exposed to cisplatin. DNA extracted from cells treated with cisplatin at indicated concentrations for 5 h, assayed by ELISA. Points, mean readings from three wells; bars indicate S.E.M. S.E.M. lie within symbols unless shown by bars).

3.3. Similarity in the binding pattern of MAb62-5 and CDRP

The endogenous nuclear proteins of cells which recognize cisplatin–DNA adducts (i.e. CDRP) have previously been identified by DNA mobility shift assay [20]. Competition patterns of MAb62-5 and CDRP binding activities were compared (Fig. 4A). Binding competition using 8 μg of nuclear extracts in the absence (lanes 2–7) or presence (lanes 8–13) of MAb62-5 was conducted. CDRP binding (indicated with an arrow) decreased with increasing amounts of competitor f103-pt: 0, 10, 100 and 1000 fold (lanes 2–5, respectively). Approximately 50–60% binding was inhibited by a 100 or 1000 fold competitor. In contrast, 1000 fold of f103 or f103-uv did not inhibit, or only slightly affected, CDRP binding (lanes 6 and 7, respectively). In the presence of MAb62-5, the competition pattern of the CDRP binding activity was essentially not affected. Under this condition (1 ng f103-pt, D/N = 0.08) the competition of the MAb62-5 binding (indicated with an arrowhead) was also unaffected by CDRP. Neither binding activities were inhibited by f103 or f103-uv (lanes 12 and 13). The binding activities MAb62-5 and CDRP were calculated (Fig. 4B). The relative binding of MAb62-5 (Ab), CDRP alone (−) or together with MAb62-5 (+) appeared to be similar. The IC_{50}, i.e. molar excess competitor causing 50% inhibition, was 50–60 for each case.

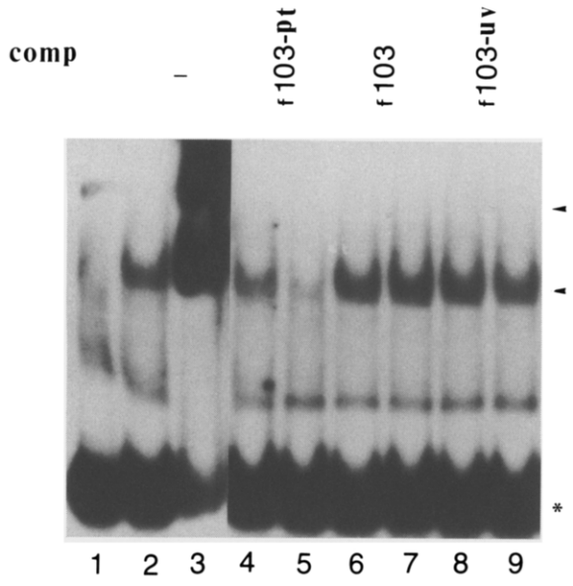

Fig. 3. Binding of MAb62-5 to cisplatin–DNA adducts as measured by DNA mobility shift assay. Exactly 1 μl of MAb62-5 at 1:25 dilution in the absence (lane 2) or presence of competitor, f103-pt (D/N = 0.08), f103 or f103-UV (6000 J/m^2), was incubated with 1 ng of DNA probe f103-pt (D/N = 0.08). Lane 1, probe alone; lane 3, MAb62-5 without dilution. The competitors are in 100 fold (lanes 4, 6 and 8) or 1000 fold molar excess (lanes 5, 7 and 9). *Free probe; arrowhead, bound probe. comp, competitor.

3.4. Reduced accumulation of cisplatin–DNA adduct in the resistant cells

A comparison of the removal kinetics of cisplatin–DNA adducts between HeLa-CPR and the parental HeLa cells was done using the ELISA assay (Fig. 5). HeLa and HeLa-CPR cells were treated, respectively, with 50 and 200 μM cisplatin for 5 h, and the amount of cisplatin–DNA adducts was measured by ELISA at 0, 4, 12 or 24 h after cisplatin treatment (Fig. 5, upper panel). It should be noted that these cisplatin concentrations caused 37% survival of each of the cell lines as determined by colony forming assay. Both repair patterns showed a slight increase, with a peak accumulation at 4 h followed by a decrease in the relative $OD_{405\ nm}$. Cisplatin-treated resistant and parental cells with a similar peak accumulation of cisplatin–DNA adducts exhibited a nearly identical kinetic pattern following repair incubation. When both cell lines were treated with 50 μM cisplatin, the patterns were similar. However, the relative $OD_{405\ nm}$ of HeLa-CPR cells is ~50% less than that of HeLa cells (data not shown). The data indicated that the removal rate of cisplatin–DNA adducts was the same in both cell lines. The dose–response curve was also determined (Fig. 5, lower panel). Cells were treated with 0, 25, 50, 75 or 150 μM of cisplatin. Following 5 h of incubation, the level of cisplatin–DNA adducts in cells was analyzed. There was a linear correlation (with indicated regression coefficient R) within these concentrations of cisplatin in both cells. The regression lines of the dose–responses were indicated with slopes of 0.0057 and 0.00276 for HeLa and HeLa-CPR cells, respectively, indicating a ~2 fold enhancement of DNA repair in HeLa-CPR cells.

3.5. Demonstration of enhanced repair in the resistant cells by indirect methods

To further evaluate results obtained by immunoassays, DNA repair in the resistant and the parental cells was measured by conventional methods (Table 2). The slopes for HeLa and HeLa-CPR cells obtained from the immunoassay were 0.0057 and 0.0028, respectively. The estimated enhancement of DNA repair was 2.04 fold. For alkaline elution [26], cells were treated with 50 μM cisplatin for 5 h, incubated for 24 h, and repair-associated DNA strand breaks were measured. The excess

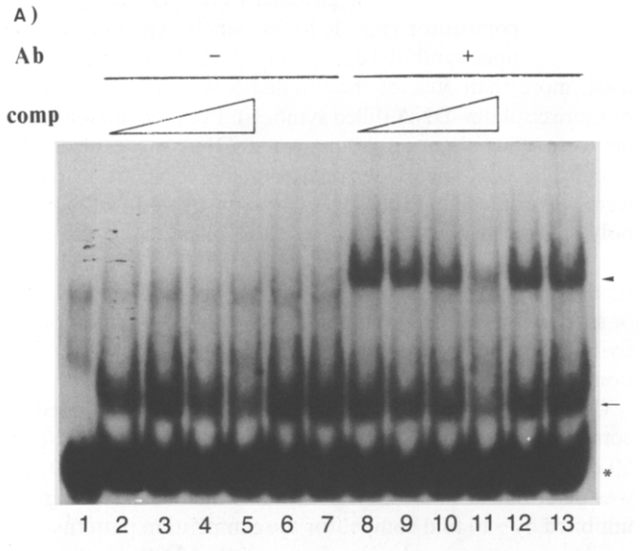

Fig. 4. Binding of nuclear extracts to cisplatin–DNA adducts. (A) Binding of 8 μg of nuclear extracts in the absence (lanes 2–7) or presence (lanes 8–13) of MAb62-5 (1 μl at 1:25 dilution). The binding reaction was conducted without (lanes 2 and 8) or with increasing amounts of competitor: 10, 100, and 1000 fold f103-pt (D/N = 0.2) (respectively for lanes 3–5 and lanes 9–11), 1000 fold f103 (lanes 6 and 12), or 1000 fold f103-uv (lanes 7 and 13). *Free probe; arrowhead, MAb62-5 bound probe; arrowhead, nuclear extracts bound probe. comp, competitor. (B) Quantitation of binding competition. Relative adduct binding vs. fold competitor was estimated by scanning densitometry of the intensity of the bound probe (data derived from A). CDRP(−), nuclear extract binding in the absence of MAb62-5; CDRP(+), nuclear extract binding in the presence of MAb62-5. Ab, MAb62-5 binding.

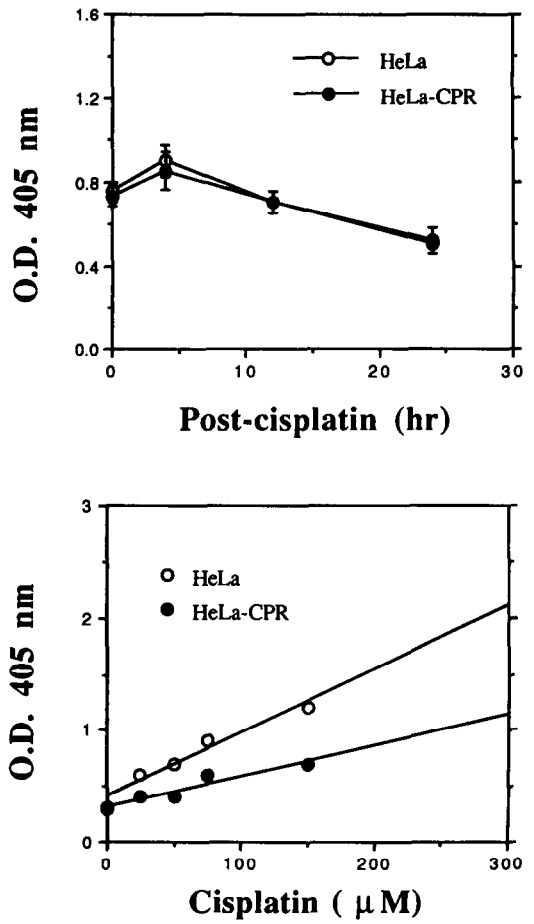

Fig. 5. Repair of cisplatin–DNA adducts in HeLa and HeLa-CPR cells as measured by ELISA. (Upper panel) Repair kinetics following cisplatin treatment. Cells were treated with cisplatin (50 and 200 μM for HeLa and HeLa-CPR cells, respectively) for 5 h to accumulate an equal level of cisplatin–DNA adducts, and incubated in drug-free culture medium for various times prior to the assay. The $OD_{405\ nm}$ (mean ± S.D.) (n = 3) of tested vs. untreated cells is shown. (Lower panel) Dose–response of cisplatin–DNA adduct accumulation. Cells were treated with various concentrations of cisplatin for 5 h, incubated for 24 h, and the relative $OD_{405\ nm}$ was determined. The lines were calculated by linear regression as $y = 0.39809 + 0.0056983x$; $R^2 = 0.952$ (HeLa) and $y = 0.31225 + 0.0027624x$; $R^2 = 0.905$ (HeLa-CPR), where R is the correlation coefficient.

DNA strand breaks of HeLa and HeLa-CPR cells vs. incubation time were determined. There were ~1.6 excess strand breaks (ESB) per 10^{10} Da of HeLa DNA following a 1 min incubation as compared to ~3.1 breaks under the same assay conditions in HeLa-CPR cells. The slopes of the regression curves were 0.99 and 0.516 for the resistant and the parental cells, respectively, indicating a 1.92 fold enhancement of the repair rate in HeLa-CPR cells. DNA repair in the resistant and the parental cells was also measured by plasmid reactivation, an indirect measurement of DNA excision repair [6]. Plasmid DNA pRSVcat with D/N = 0, 0.001, 0.002, or 0.004 was co-transfected with un-treated pSVβ for transient expression. The CAT and β-galactosidase activities were measured 40 h after transfection. The IC_{50}, i.e. cisplatin concentration inhibiting 50% CAT activity, for HeLa and HeLa-CPR cells were D/N = 0.0007 and 0.0015, respectively. The IC_{50}, as determined by the plasmid reactivation assay for HeLa and HeLa-CPR cells, corresponded to ~3.5 and 7.5 cisplatin per plasmid, respectively. There was a ~2.1 fold enhancement of the plasmid reactivation in the resistant cells. Thus, the enhanced DNA repair in HeLa-CPR cells, as measured by our immunoassay, is comparable to the results obtained by conventional methods.

4. Discussion

In this study, a monoclonal antibody was prepared for the measurement of cisplatin–DNA adducts. According to the competition pattern of ELISA, in vitro, 50% of MAb62-5 binding was inhibited by 7 ng of competitor (Fig. 1), which corresponded to 0.21 μM in a typical 100 μl reaction (1 μg/ml = 3 μM). Competitive ELISA in vivo demonstrated that MAb62-5 preferentially interacted with cisplatin–DNA adducts. Immunofluorescence microscopy analysis also indicated that the antibody was specific to the nucleus, but not the cytosol, in cells treated with cisplatin (data not shown). Using cisplatin–DNA (D/N = 0.03) as an immunogen, Lippard and co-workers reported a monoclonal antibody the binding activity of which was inhibited by 4–6 nM (50% inhibition) but appeared to be insufficiently sensitive for biologial experiments [22]: it was 42 fold less sensitive in MAb62-5 immunoreactivity. In an in vivo study, Tilby et al. [35] demonstrated an immunoassay in Walker tumor cells based upon a monoclonal antibody that enabled the quantitation of cisplatin–DNA adducts down to 3 nmol cisplatin/g DNA (i.e. 1 cisplatin adduct/10^6 bases). We also detected DNA adducts from 15 μM cisplatin-treated HeLa cells, which corresponded to 342.48 nmol cisplatin/g DNA or 8.8 cisplatin adduct/10^3 bases, determined by atomic absorption spectrophotometry [35]. Furthermore, DNA adducts could also be detected from cells treated with 5 μM cisplatin (210 nmol cisplatin/g DNA or 1.4 adducts/10^4 bases). This value was ~70 fold lower than that reported by Tilby et al. [36]. Although we did not measure the low limit of MAb62-5 sensitivity, it is

Table 2
Comparison of DNA repair measured by different methods in HeLa and the resistant HeLa-CPR cells

	HeLa	HeLa-CPR	Fold alteration[d]
Immunoassay (AFF)[a]	0.0057 ± 0.0006	0.0028 ± 0.0004	2.04
Alkaline elution (ESB)[b]	0.516 ± 0.051	0.99 ± 0.1	1.92
Plasmid reactivation, IC_{50} (D/N)[c]	0.0007 ± 0.00012	0.0015 ± 0.0003	2.14

[a] The data were expressed as AFF ± S.D. (n = 3). AFF, average adduct formation frequency, defined by $OD_{405\ nm}/\mu$M cisplatin as assayed by ELISA (also see Fig. 2). The background AFF has been substracted from the values shown.
[b] The data were expressed as ESB ± S.D. (n = 3). ESB, average excess strand breaks, per 10^{10} nucleotides genomic DNA per min incubation.
[c] The data were expressed as IC_{50} ± S.D. (n = 3), estimated cisplatin–DNA adducts (D/N) required to inhibit 50% CAT activity, as assayed by plasmid reactivation.
[d] Fold alteration was calculated as fold decrease in immunoassay, and fold increase in alkaline elution and plasmid reactivation assays.

probably similar to the previously reported antibody [36]. If MAb62-5 showed reduced sensitivity, it could be explained by the suggestion that an immunogen with a high level of D/N modification causes low efficiency in detection [37]. This is supported by the preparation of a monoclonal antibody with good sensitivity for cisplatin–DNA adducts using lowered cisplatin/DNA as an immunogen [38]. In this study, we have demonstrated the feasibility of using MAb62-5 to differentiate cisplatin–DNA adducts accumulated in the resistant and the parental cells. The data was consistent with measurement by alkaline elution and plasmid reactivation assays. Enhanced DNA repair detected in HeLa-CPR cells suggested a causative mechanism(s) for the resistant phenotype. The kinetic pattern of the accumulation of cisplatin–DNA adducts in the resistant cells is the same as in the parental cells except that the initial level of cisplatin–DNA adducts is 50% lower. Therefore, the reduced frequency of adduct formation plays a major role in the overall enhancement of DNA repair in the resistant cells. To verify the possible genetic alterations, a revertant subline, HeLa-rev, derived from HeLa-CPR, was characterized [39]. The IC_{50} of DNA repair using the immunoassay as described above was 0.0039, giving a 1.2 fold increase in DNA repair compared to HeLa cells. The results suggested that the phenotypic change in resistance was due to a simple mutation. We have previously demonstrated that the cross-resistance of HeLa-CPR cells to UV damage is associated with enhanced recognition and incision of UV–DNA adducts in a cell-free system [21]. Current studies have demonstrated the improved excision repair of cisplatin–DNA adducts in the resistant cells, which is also largely determined by the early stage of the repair process. Since reduced repair synthesis in XP group A cells has been assigned to the incision step [40], our resistant cells have most likely acquired an enhanced DNA repair that is deficient in the XP cells.

The specificity of MAb62-5 was also confirmed by the DNA mobility shift assay in vitro. 50% inhibition of the MAb62-5 binding required 50 fold platinated-DNA competitor, whereas less than 5% binding was inhibited by 1000 fold UV-irradiated DNA. Interestingly, it showed a similar affinity to platinated DNA between the antibody and the endogenous cisplatin DRP in cells. In contrast, 50% inhibition of the UVDRP binding activity required only ~20 fold excess of specific competitor [41]. Therefore, MAb62-5 as well as cisplatin DRP distinguishes ciplatinum modification from UV modification of the same DNA molecule. The sensitive detection of cisplatin–DNA adducts in vivo using monoclonal antibody has also been demonstrated by others [36]. Assays with apparently reduced sensitivities at low DNA modification levels have been described for other types of DNA damage [42–44]. This may be partly explained by the presence of CDRP in cells, which potentially competes with the antibody. As demonstrated in this study, MAb62-5 showed a similar affinity to the HeLa CDRP for cisplatin-modified plasmid DNA, suggesting that the binding domain of MAb62-5 is homologous to that of CDRP. It is likely that a low level of cisplatin–DNA adducts in the cellular chromosome is bound by endogenous CDRP prior to assessment and not by the antibody during immunochemical detection. However, one may underestimate the amount of cisplatin–DNA adducts using immunoassay due to competitive pre-occupation by endogenous CDRP. Therefore, one should be cautious when comparing cisplatin–DNA repair between different cell lines using the immunoassay because variation of CDRP in cells may complicate the immunochemical detection. Auxiliary to this is the underestimation of cisplatin–DNA adducts using antibody in cells harboring a high CDRP level. The simple DNA mobility shift assay of CDRP in vitro is informative in evaluating the in situ detection of cisplatin–DNA adducts using antibodies. The same strategy may be applied to explain the complexicity using the immunoassay for other DNA adducts such as UV-damaged DNA.

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References

[1] Loehrer, P. and Einhorn, L.H. (1984) Cisplatin. Ann. Intern. Med. 100, 704–713.
[2] Rosenberg, B. (1985) Cancer (Phila.) 55, 2303–2316.
[3] Hospers, G.A.P., Mulder, N.H. and De Vries, E.G.E. (1988) Med. Oncol. Tumor Pharmacother. 5, 145–151.
[4] Roberts, J.J. and Thomson, A.J. (1979) Prog. Nucleic Acid Res. Mol. Biol. 22, 71–133.
[5] Chu, G. and Berg, P. (1987) Mol. Biol. Med. 4, 277–290.
[6] Chao, C.C.-K., Lee, Y.-L., Cheng, P.-W. and Lin-Chao, S. (1991) Cancer Res. 51, 601–605.
[7] Eastman, A. and Richon, V.M. (1986) in: Biochemical Mechanisms of the Platinum Antitumor Drugs (McBrien, D.C.H. and Slater, T.F., eds.) pp. 91–119, IRL Press, Oxford, England.
[8] Wood, R.D., Robins, P. and Lindahl, T. (1988) Cell 53, 97–106.
[9] Wood, R.D. (1989) Biochemistry 28, 8287–8292.
[10] Hansson, J. and Wood, R.D. (1989) Nucleic Acids Res. 17, 8073–8091.
[11] Sibghat-Ullah, Husain, I., Carlton, W. and Sancar, A. (1989) Nucleic Acids Res. 17, 4471–4484.
[12] Sibghat-Ullah and Sancar, A. (1990) Biochemistry 29, 5711–5718.
[13] Hansson, J., Munn, M., Rupp, W.D., Kahn, R. and Wood, R.D. (1989) J. Biol. Chem. 264, 21788–21792.
[14] Hansson, J., Keyse, S.M., Lindahl, T. and Wood, R.D. (1991) Cancer Res. 51, 3384–3390.
[15] Robins, P., Jones, C.J., Biggerstaff, M., Lindahl, T. and Wood, R.D. (1991) EMBO J. 10, 3913–3921.
[16] Chu, G. and Chang, E. (1988) Science 242, 564–567.
[17] Kataoka, H. and Fujiwara, Y. (1991) Biochem. Biophys. Res. Commun. 175, 1139–1143.
[18] Chao, C.C.-K. (1992) Biochem. J. 282, 203–207.
[19] Chao, C.C.-K., Huang, S.-L., Huang, H.-M. and Lin-Chao, S. (1991) Mol. Cell. Biol. 11, 2075–2080.
[20] Chao, C.C.-K., Huang, S.-L., Lee, L.-Y. and Lin-Chao, L.-Y. (1991) Biochem. J. 277, 875–878.
[21] Chao, C.C.-K. and Huang, S.-L. (1993) Mutat. Res. 303, 19–27.
[22] Sundquist, W.I., Lippard, S.J. and Stollar, B.D. (1987) Proc. Natl. Acad. Sci. USA 84, 8222–8229.
[23] Skoog, D.A. and Leary, J.J. (1992) in: Principles of Instrumental Analysis, pp. 211–223, Holt Saunders, Philadelphia.
[24] Poirier, M.C., Lippard, S.J., Zwelling, L.A., Ushay, H.M., Kerrigan, D., Thill, C.C., Santella, R.M., Grunberger, D. and Yuspa, S.H. (1982) Proc. Natl. Acad. Sci. USA 79, 6443–6447.
[25] Malfoy, B., Hartmann, B., Macquet, J.P. and Leng, M. (1981) Cancer Res. 41, 4127–4131.
[26] Chao, C.C.-K. and Rosenstein, B.S. (1986) Photobiochem. Photobiol. 43, 165–170.
[27] Rosenstein, B.S. and Ducore, J.M. (1983) Photochem. Photobiol. 38, 51–55.
[28] Chu, G., Hayakawa, H. and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326.
[29] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[30] Ushay, H.M., Tullius, T.D. and Lippard, S.J. (1981) Biochemistry 20, 3744–3748.

[31] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[32] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[33] Lammli, U.K. (1970) Nature 227, 680–685.
[34] Haunnighausen, L. and Lubon, H. (1987) Methods Enzymol. 152, 721–735.
[35] Chao, C.C.-K. (1994) Mol. Pharmacol. 45, 1137–1144.
[36] Tilby, M.J., Johnson, C., Knox, R.J., Cordell, J., Roberts, J.J. and Dean, C.J. (1991) Cancer Res. 51, 123–129.
[37] Fichtinger-Schepman, A.-M.J., Baan, R.A. and Berends, F. (1989) Carcinogenesis 10, 2367–2369.
[38] Terheggen, P.M.A.B., Floot, B.G.J., Scherer, E., Begg, A.C., Fichtinger-Schepman, A.-M.J. and Den Engelse (1987) Cancer Res. 47, 6719–6725.
[39] Chao, C.C.-K. (1994) Eur. J. Pharmacol. (in press).
[40] Cleaver, J.E. and Kraemer, K.H. (1989) in: The Metabolic Basis of Inherited Disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds.) pp. 2949–2971, McGraw-Hill, New York.
[41] Chao, C.C.-K., Huang, S.-L. and Lin-Chao, S. (1991) Nucleic Acids Res. 19, 6413–6418.
[42] VanSchooten, F.J., Kriek, E., Steenwinkel, M.-J.S.T., Noteborn, H.P.J.M., Hillebrand, M.J.X. and Van Leeuwen, F.E. (1987) Carcinogenesis 8, 1263–1269.
[43] Santella, R.M., Weston, A., Perera, F.P., Trivers, G.T., Harris, C.C., Young, T.L., Nguyen, D., Lee, B.M. and Poirier, M.C. (1988) Carcinogenesis 9, 1265–1269.
[44] West, G.J., West, I.W.-L. and Ward, J.F. (1982) Radiat. Res. 90, 595–608.