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Advances in Brief

Elevated DNA Repair Capacity Is Associated with Intrinsic Resistance of Lung Cancer to Chemotherapy[1]

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Abstract

Non-small cell lung cancer (N-SCLC) is generally unresponsive to chemotherapy even without previous drug treatment, as opposed to small cell lung cancer (SCLC), which is initially responsive to chemotherapy. The mechanisms of this intrinsic resistance are unknown. This study was designed to investigate the role of DNA repair in intrinsic resistance of N-SCLC to cisplatin. A panel of primary N-SCLC cell cultures and established cell lines were examined and compared to SCLC cell lines established previously from untreated patients. The overall DNA repair capacity was estimated by the ability of cells to reactivate the pRSV-CAT plasmid damaged by cisplatin ("host cell reactivation" assay). Cytotoxicity was determined for cisplatin *in vitro*. N-SCLC cells were found to be significantly more resistant to cisplatin than SCLC cell lines isolated from untreated patients ($P < 0.01$). The capacity of N-SCLC cells to reactivate pRSV-CAT plasmid damaged with cisplatin and transfected into cells was higher in N-SCLC cells than in SCLC cells originating from patients who were untreated previously ($P < 0.05$). Correlation was also observed between chloramphenicol acetyltransferase activity and intrinsic resistance to cisplatin. However, no significant difference was observed between primary N-SCLC cultures and established cell lines. This study indicates that elevated DNA repair capacity is associated with drug resistance in lung cancer and suggests that modulation of DNA repair mechanism(s), such as the incorporation of specific DNA repair inhibitor(s) in therapeutic regimens, may help to improve therapeutic strategies of N-SCLC.

Introduction

Lung cancer is a leading cause of cancer mortality in men worldwide (1). Its incidence is also increasing in women as a consequence of smoking, and, as a result, lung cancer is becoming a major cause of mortality in females.

Lung cancer is divided into two major groups, N-SCLC[3] and SCLC, each having distinct histopathological, phenotypical, and genotypical characteristics. N-SCLC, which refers mainly to adenocarcinoma, squamous cell carcinoma/epidermoid, and large cell carcinoma, accounts for approximately 80% of total lung cancer cases. Most N-SCLC patients have locally advanced and unresectable disease or metastasis at their initial diagnosis. Furthermore, a significant number of patients with resectable cancer develops a recurrence after surgery (2). In all cases, these patients are treated with chemotherapy regimens, particularly those containing CDDP. Unfortunately, this treatment results in only a limited improvement in overall survival, and the 5-year survival rate remains lower than 10% once the disease has disseminated (2). One of the major causes for this limitation of treatment is the unresponsiveness of N-SCLC to drugs, even without previous treatment, as opposed to SCLC, which is initially responsive to chemotherapy. The mechanisms of this intrinsic resistance are unknown.

None of the cellular mechanisms found to be associated with acquired resistance have been shown to correlate with drug response in N-SCLC (3). Gazdar's group reported (4) that other features of N-SCLC, such as the presence of neuroendocrine markers and overexpression of the *HER-2/neu* gene (but not *ras* oncogene; Ref. 5) were associated with the chemosensitivity of N-SCLC. The molecular basis for these observations is still unknown. Another mechanism that has not been investigated in lung cancer is DNA repair. This process is a ubiquitous defense mechanism essential for cell survival. An increase in DNA repair capacity leads to resistance to the genotoxic effect of drugs and carcinogens, whereas its decrease potentiates drug toxicity but also increases gene instability and susceptibility to mutations. Increased repair capacity has been shown to be associated with drug-acquired resistance in various cell lines and clinical specimens (6–11).

In this study we have investigated the capacity of primary and established N-SCLC cells to reactivate the pRSV-CAT plasmid damaged by CDDP. The pRSV-CAT plasmid contains the bacterial gene coding for CAT under the control of the RSV long-terminal repeat promoter, which is highly expressed in mammalian cells (12). Platination of the pRSV-CAT plasmid will diminish or abolish *CAT* gene expression, as a consequence of DNA damage, after transfection into cells. Repair of these lesions will restore *CAT* gene expression and provide information about the overall repair capacity of a given cell population. This assay, referred to as the "host cell reactivation" or "CAT" assay, has been used extensively to compare repair of DNA damage in xeroderma pigmentosum cells, in sensitive and resistant cells (13, 14), in p53 proficient and deficient cells (15), and also to evaluate environmental exposure to various carcinogens in skin and peripheral blood (16, 17).

We have also investigated the relationship between the capacity of N-SCLC cells to reactivate the CDDP-damaged pRSV-CAT plasmid and cell sensitivity to CDDP.

Materials and Methods

Materials. CDDP was obtained from David Bull Laboratories, Inc., (Vaudrevil, Quebec, Canada), at a concentration of 1 mg/ml in saline. The pRSV-CAT plasmid was obtained from the ATCC; pRSV-β-galactosidase plasmid was obtained from Dr. J. Hiscott (Lady Davis Institute, Montreal, Quebec, Canada); *Escherichia coli* CAT and acetyl coenzyme A were obtained from Pharmacia; and D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol (specific activity 53.1 mCi/mmol) was obtained from Amersham.

Tumor Specimens and Primary Cell Culture. Fresh surgical specimens were washed with Hanks' balanced salt solution (HBSS) containing 1% gentamicin. Their histological type was confirmed by pathological examination before processing. Slices of approximately 0.5 mm were removed from

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[3] The abbreviations used are: N-SCLC, non-small cell lung cancer; AUC, area under the curve; CAT, chloramphenicol acetyltransferase; CDDP, cisplatin (*cis*-diamminedichloroplatinum II); SCLC, small cell lung cancer; ATCC, American Type Culture Collection; IC_{50}, 50% inhibitory dose.

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non-necrotic tumor tissue, washed with complete media (either DMEM-F12, α-MEM, or RPMI), supplemented with nonessential amino acids, 5 μg/ml insulin, 10 μg/ml transferrin, 10^{-8} M hydrocortisone, 1 ng/ml epidermal growth factor, 2×10^{-8} M sodium selenite, and 0 to 2% serum. Culture media that promoted cell growth was selected as standard. Cells were placed in 6-well plates on a scratched surface to facilitate the fixation. Duplicate cultures were initiated on wells precoated with a 1-ml solution containing 10 μg human fibronectin, 10 μg collagen, and 5 μg BSA. A drop of complete media was added to each explant. After 4 h, explants were carefully covered with media and incubated at 37°C in 5% CO_2. Media were changed after 24 h and every 2 days thereafter. When significant outgrowth of tumor cells radiated from the tissue onto the surface of the dish, explants were transferred to new dishes to initiate another culture. Outgrown cells were either kept in culture or dissociated into single cells with trypsin-EDTA. During dissociation, cells were incubated with the enzyme for a brief 1–3 min at 37°C. Fibroblasts (if present) remained adherent to the plastic, whereas most tumor cells attached poorly during this period. The suspension was centrifuged at 500 × *g* for 5–10 min, and the pellet was resuspended in complete media. In some cases, cell suspension was obtained by mechanical or enzymatic (collagenase-trypsin-hyaluronidase) digestion. Cells from different tissues were grown in 6-well plates to 70–80% confluence and then used for transfection studies. To compare N-SCLC and SCLC, we have also used a panel of N-SCLC and SCLC cells obtained from the ATCC (Table 1). SCLC cells consisted of NCI-H209, NCI-H187, NCI-H889, (classical SCLC isolated from untreated patient), and NCI-H417 (variant SCLC isolated from untreated patient). All these cells were maintained in the corresponding culture media as referred by the ATCC.

Cytotoxicity. Cytotoxicity was determined for CDDP by using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (18), with the exception that cells were seeded at a concentration of $1–5 \times 10^3$ cells/well, and incubated with the drug for 96 h. CDDP was used at concentrations ranging from 0.01 to 20 μM. The IC_{50} was calculated as the dose of the drug causing a 50% reduction in absorbance compared to control cells treated with the vehicle alone.

Host Cell Reactivation Assay. pRSV-CAT was amplified in an *E. coli* strain, and DNA was prepared by alkaline lysis and cesium chloride/ethidium bromide ultracentrifugation. The form I plasmid DNA was isolated by a cesium chloride gradient centrifugation, treated with RNase, and purified to ensure that >90% was in supercoiled form. Plasmid DNA was dissolved in 3 mM NaCl-1 mM sodium phosphate (pH 7.4) and incubated with CDDP at 37°C for 24 h in the dark, at a plasmid concentration of 100 μg/ml (3×10^{-4} M nucleotide phosphate). Final CDDP concentrations varied between 1 and 10 μM. After 24 h, the concentration of NaCl was increased to 0.2 M in order to quench CDDP adducts, and DNA was collected by two consecutive precipitations with absolute ethanol and washes with 70% ethanol. DNA was dissolved in 10 mM Tris-HCl-1 mM EDTA (pH 7.4), aliquoted, and stored at −80°C until transfection. Cells at 0.5 to 3×10^6/well were seeded in 6-well plates, and transfection was initiated when cells reached approximately 80% confluence.

Five to ten μg of pRSV-CAT DNA, 2 μg of pRSV-β-galactosidase plasmid, and 10 μg of lipofectin (GIBCO-BRL) were mixed gently in a sterile polystyrene tube in a final volume of 200 μl of serum-free medium, and incubated for 30 min at 37°C. This mixture was then added to 1.8 ml of media containing 1% serum and layered over the cells; the cells were transfected in 2-ml ependorff tubes. Eighteen h later, cotransfection was stopped by removing the liposome-DNA mixture, washing cells twice with media, followed by the addition of complete media. The cells were then cultured in 6-well plates in normal media. After 48-h incubation, cells were transferred to microcentrifuge tubes, washed with PBS, and resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.8. Cells were then lysed by three consecutive freeze-thawing cycles (5 min each). The lysate was centrifuged at 14,000 × *g* for 5 min at 4°C. Aliquot of the supernatant was heated at 65°C for 7 min to destroy deacetylase activity (13) and used to determine CAT activity or stored at −80°C for subsequent experiments. Another aliquot was used to determine β-galactosidase activity. Protein concentration was determined by the Lowry method (19).

The CAT reaction mixture [40 μl of 0.25 M Tris-HCl (pH 7.6); 20 μl of 4 mM acetyl coenzyme A; 50 μg of cell extract protein; and 70 μl of [^{14}C]chloramphenicol (a final concentration of 10 μM) in a final volume of 160 μl] was vortexed briefly, centrifuged, and incubated at 37°C for 6–8 h. The mixture was extracted once with 1 ml 100% ethyl acetate and then centrifuged for 1 min. The organic phase was collected, dried in a speed vacuum, redissolved in 10 μl ethylacetate, and loaded on TLC plates. After 30–45 min migration in a TLC chamber saturated with chloroform:methanol (95:5, v/v), the plates were dried, and subjected to autoradiography. The radioactivity of chloramphenicol and its metabolites was determined by scintillation counting. SCLC cell lines were transfected in suspension under similar conditions described above. pRSV-neo transfected cells, which were used as a negative control, expressed no CAT activity. Excision repair-deficient Chinese hamster cell line UV20 (complementation group 1) was used as a control for repair deficiency.

Preliminary transfection experiments were performed in each cell line to ensure the amount of pRSV-CAT DNA used for transfection studies give efficiency in the linear range. Protein concentration was also determined to be in the linear range. A linear standard curve was also obtained with pure CAT by using a wide range from a 0.1 to 100 units ($r > 0.95$).

The activity of β-galactosidase was determined spectrophotometrically as described (20). Relative CAT activity was determined as: (percentage of CAT conversion/50 μg protein)/(β galactosidase activity/50 μg protein).

For comparative purposes, we have calculated, from CAT activity curves, the AUC for each cell line using the "Kaleidagraph" Macintosh program.

Statistical Analysis. The mean values of IC_{50} and CAT activity were compared by a two-tailed Student's *t* test, and the ANOVA was compared with intergroup comparisons [by using the "Statworks" program (Abacus Concepts, Inc.,)].

Table 1 *Lung cancer cell lines*

Cancer type	Cell line	Source	Prior treatment	Origin
N-SCLC				
ADC[a]	L13[b]	Primary tumor	UT[c]	This laboratory
	L15	Primary tumor	UT	This laboratory
	L16	Primary tumor	UT	This laboratory
	NCI-H596[c]	Primary tumor	XR	ATCC
SQ	L3	Primary tumor	UT	This laboratory
	L4	Primary tumor	UT	This laboratory
	NCI-H520	Primary tumor	UT	ATCC
	FADU	Primary tumor	UT	ATCC
LCC	NCI-H460	Pleural effusion	UT	ATCC
	NCI-H661	Lymph node	UT	ATCC
SCLC-UT	NCI-H209	Bone marrow	UT	ATCC
	NCI-H187	Pleural effusion	UT	ATCC
	NCI-H889	Pleural effusion	UT	ATCC
	NCI-H417	Pleural effusion	UT	ATCC

[a] ADL, adenocarcinoma; SQ, squamous carcinoma; LCC, large cell carcinoma.
[b] L13, L15, L16, L3, and L4 are primary cell cultures established from fresh specimens.
[c] UT, untreated; XR, treated with X-ray radiation.
[d] The prefix NCI was omitted in the text and figures for simplicity.

Results

Primary cell cultures L3, L4, L13, L15, and L16 were established from clinical specimens isolated from untreated N-SCLC patients. Their histopathological classification is summarized in Table 1. L3 and L4 were derived from squamous carcinomas of stage I (T_2) resected from male patients. L13, L15, and L16 were derived from adenocarcinomas of stage I (T_2; L13 and L15) and stage II (T_2; L16) resected from female patients. The degree of differentiation varied from moderately differentiated (L3 and L15) to poorly differentiated (L4, L13, and L16). Patients were between 57 and 70 years old and were either active or passive smokers. Examination by phase contrast microscopy showed adherent cells with an epithelial-like structure, growing either as aggregates or as individual cells. All primary cells used for CAT were at passage 2 (L13 and L4), passage 3 (L3 and L15), and passage 6 (L16). The characteristics of the other cell lines are summarized in Table 1.

The cytotoxic effect of CDDP on N-SCLC primary cell cultures and cell lines growing in the exponential phase was compared to the effect on SCLC cell lines previously established from untreated patients (Table 1). As indicated in Fig. 1, N-SCLC cells were signifi-

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cantly more resistant to CDDP than were SCLC cell lines ($P < 0.01$). There was approximately a 2–4 fold greater resistance to CDDP in N-SCLC as compared to SCLC.

The amount of CDDP binding to pRSV-CAT DNA has been shown to be proportional to the initial CDDP concentration in the incubation buffer (13, 21, 22). This is evident by the fact that increasing CDDP concentration from 1 to 10 μM (giving an average number of 3–12 platinum adducts/plasmid kb pair as determined by atomic absorption spectroscopy, respectively) is proportional to the CAT inhibition in our experiments (Fig. 2). Higher concentrations completely abolished CAT activity in all cells tested (data not shown). Comparison of the AUC (Table 2) showed that a significant difference exists between N-SCLC and SCLC cells tested ($P < 0.01$). The Chinese hamster

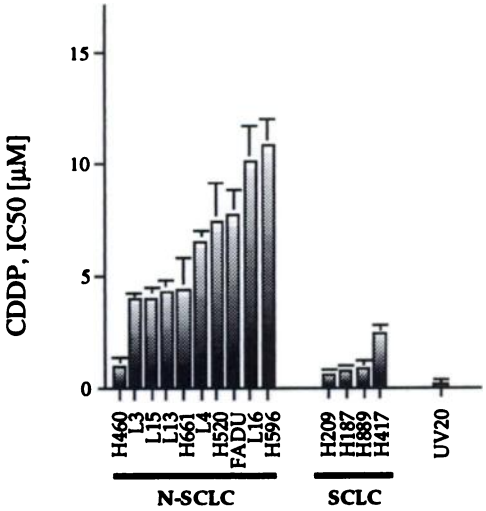

Fig. 1. Cytotoxic effect of CDDP in N-SCLC and SCLC cells. IC_{50}s were determined from the dose-response curve for each cell line. *Columns*, average of four independent determinations; *bars*, ± SD. N-SCLC *versus* SCLC; $P < 0.01$.

Table 2 *Host cell reactivation capacity in N-SCLC and SCLC cells*

Cell line	Transfection efficiency[a] Mean ± SD	AUC[b] Mean ± SD
Adenocarcinoma		
L13	57.7 ± 7.5	670.3 ± 37.2
L15	66.0 ± 8.7	730.8 ± 21.9
L16	61.0 ± 10.0	783.2 ± 11.7
H596	76.5 ± 6.5	731.0 ± 13.8
Squamous		
L3	66.7 ± 6.6	561.7 ± 8.6
L4	71.4 ± 10.2	679.5 ± 12.9
H520	70.1 ± 1.0	654.9 ± 18.9
FADU	69.0 ± 7.3	673.8 ± 9.7
Large cell carcinoma		
H661	61.2 ± 7.3	756.0 ± 21.1
H460	50.0 ± 7.7	396.8 ± 27.7
SCLC-UT		
H417	66.7 ± 8.5	497.9 ± 11.2
H889	65.6 ± 4.24	493.9 ± 56.1
H209	50.8 ± 10.5	463.0 ± 28.4
H187	75.3 ± 11.0	522.4 ± 9.7
UV20	85.0 ± 8.8	219.3 ± 23.1

[a] Transfection efficiency was calculated as (total CAT/parental chloramphenicol and acetylated metabolites) × 100.

[b] AUC, calculated from CAT activity curves in which CAT activity was corrected to the amount of β-galactosidase, as described in "Materials and Methods."

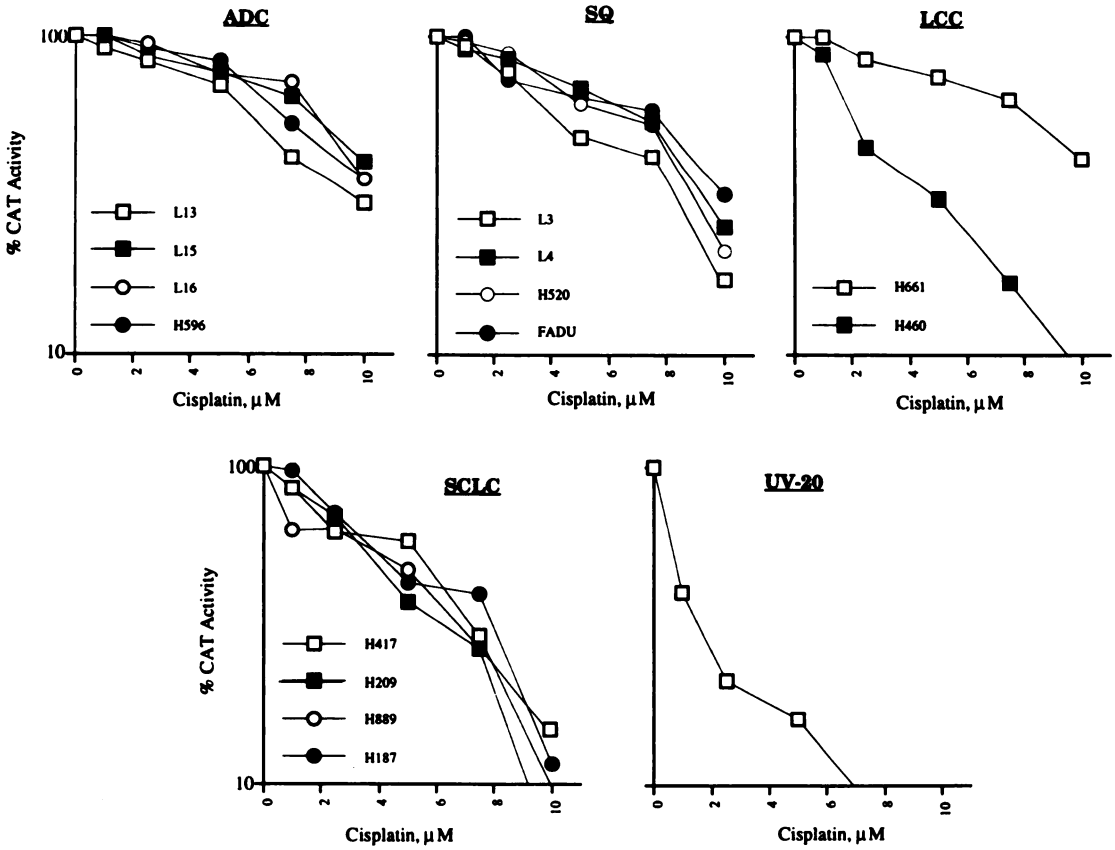

Fig. 2. Host cell reactivation capacity of N-SCLC and SCLC cells. CAT activity as related to various CDDP concentrations. Cells were cotransfected with pRSV-CAT plasmid pretreated with CDDP and untreated pRSV-β-gal as described in "Materials and Methods." *Points*, average of three independent determinations. *ADC*, adenocarcinoma; *SQ*, squamous carcinoma; *LCC*, large cell carcinoma.

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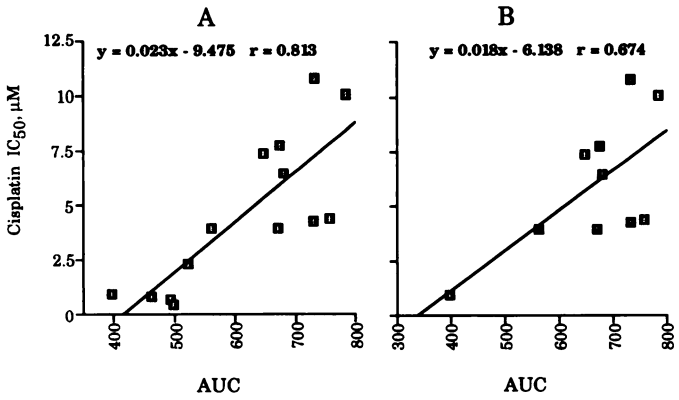

Fig. 3. Correlation of CAT activity to cytotoxicity of CDDP in N-SCLC and SCLC cells. *Points*, average of at least three independent experiments. AUC was calculated from the CAT activity curve for each cell line. *A*, all N-SCLC and SCLC cells combined; *B*, only N-SCLC cells are represented.

ovary DNA repair-deficient cell line UV20 (belonging to complementation group 1 and lacking a functional DNA excision repair gene, *ERCC1*) has the lowest CAT activity and is hypersensitive to CDDP (Table 2; Fig. 2). Comparison between CAT activity and cell sensitivity to CDDP *in vitro* indicates a good correlation between resistance and elevated CAT activity (Fig. 3).

Discussion

To investigate the role of DNA repair in the resistance of N-SCLC to chemotherapy, we have adopted the host cell reactivation assay (also referred to as the CAT assay) to estimate the intrinsic repair capacity in primary and established N-SCLC cells *versus* SCLC cells established from untreated patients.

The CAT assay is based on the capacity of cells to restore the level of expression of a nonreplicating recombinant plasmid DNA containing a CDDP-damaged *CAT* reporter gene; thus, the assay reflects the entire multistep repair process. The reliability and advantages of this assay have been reported previously (12–13, 16–17). Because the transfecting DNA is damaged before transfection into the host cells, the CAT assay permits discrimination between DNA repair and other mechanisms of resistance operative in host cells, such as abnormalities in cell membrane transport, intracellular metabolism by phase I/II enzymes, or possible inhibition/induction of a specific enzyme in host cells as a consequence of drug toxicity. Therefore, the CAT assay measures unperturbed DNA repair. However, a number of other parameters have been taken into consideration in order to compare various cell lines. These included the stability of DNA damage in plasmid DNA between various cells, transfection efficiency, and the extent of cell proliferation. CDDP-induced DNA adducts are chemically stable in contrast to adducts induced by other drugs, such as nitrogen mustards (8). No apurinic sites or breaks in platinated pRSV-CAT were detected under the treatment used (data not shown). A 24-h incubation ensures that most monoadducts are converted to intra-/interstrand cross-links. Previous reports have shown that the CDDP-treated plasmid is very stable within the cellular compartment, even at high concentrations (13). At the end of transfection, cells were confluent, and it is unlikely that differences in cell proliferation, or residual cells engaged in protein or DNA synthesis, would explain differences observed in CAT activity. Transfection efficiency was optimized to approximately 60% (in all cases, CAT values were normalized to the β-galactosidase activity of pRSV-β-gal, a plasmid used as an internal control for transfection efficiency). In view of the above arguments, our data clearly show that differences found in host cell reactivation are related to repair capacity rather than to artifacts in experimental conditions. The low plasmid reactivation obtained with the DNA repair-deficient cell line UV20 (Table 2) also supports a correlation between host cell reactivation efficiency and the overall DNA repair capacity.

Our results indicate that primary, as well as selected N-SCLC cells, have a higher capacity to repair damaged pRSV-CAT than do SCLC cells derived from patients untreated previously, and this correlates with CDDP cytotoxicity *in vitro*. Differences in CAT activity between different N-SCLC cells may be attributed to a number of variables, such as individual variation, clinical history of patients from which cells were derived, tissue and cell heterogeneity, cell histological type, and degree of cell differentiation. The low repair capacity of the N-SCLC cell line H460 may be due to a specific alteration of a DNA nucleotide excision repair gene(s), which may explain its hypersensitivity to CDDP (23). However, the H460 cell line also expresses neuroendocrine markers, which were shown previously to be associated with drug sensitivity in N-SCLC (4). Screening for DNA repair in additional N-SCLC cell lines with and without neuroendocrine marker expression will help to examine which of these mechanisms is critical for drug response.

The elevated host cell reactivation is clearly suggestive of the greater capacity of N-SCLC cells to recognize DNA damage, proceed to the incision of the damaged site, and to synthesize a new strand (24). This hypothesis is supported by previous data indicating that drugs that interfere with DNA repair synthesis, such as Gemcitabine, potentiate the cytotoxic effect of CDDP in N-SCLC (25). The mechanism(s) of elevated host cell reactivation in N-SCLC is still unknown but may involve either (*a*) up-regulation of nucleotide excision repair, a multistep repair pathway by which cells cope with a variety of DNA damage, including bulky adducts induced by CDDP, or (*b*) up-regulation of repair of CDDP-induced DNA cross-links by a recombination pathway as has been suggested for repair of DNA cross-links induced by bifunctional alkylators (26). The fact that the nucleotide excision repair genes *ERCC-1* and *ERCC-4* are homologues of *RAD10* and *RAD1*, two yeast genes involved in a yeast recombination pathway (27), respectively, suggests that there may also be an overlap between nucleotide excision repair and recombination for repair of DNA damage. Additional studies are required to investigate the role of these mechanisms in detail.

Finally, the frequent overexpression of c-*erb*B-2 (*HER2/neu*) in N-SCLC cells, as opposed to SCLC, has been shown to correlate with intrinsic drug resistance (5, 28). A previous report, using human breast cancer cells overexpressing c-*erb*B-2, showed that agonistic antibodies against the c-*erb*B-2 product, $p185^{HER2/neu}$, sensitize cells to the toxic effect of CDDP by a mechanism that is independent of drug transport or inactivation but that involves a down-regulation of DNA repair (29). This suggests that NER may be regulated at least in part by a c-*erb*B-2-coupled signaling pathway(s). Therefore, additional studies should help to clarify whether the overexpression of c-*erb*B-2 in N-SCLC is correlated with elevated DNA repair capacity in our cell panel.

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