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EFFECT OF v-*ras*^{H} ON SENSITIVITY OF NCI-H82 HUMAN SMALL CELL LUNG CANCER CELLS TO CISPLATIN, ETOPOSIDE, AND CAMPTOTHECIN

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Abstract—Expression of v-*ras*^{H} in NCI-H82 human small cell lung cancer (SCLC) cells results in a line (NCI-H82*ras*^{H}) with a non-small cell phenotype (Mabry *et al., Proc Natl Acad Sci USA* **85:** 6523–6527, 1988). This v-*ras*^{H}-associated phenotypic change is prevented by treatment with *trans*-retinoic acid (tRA) (Kalemkarian *et al., Cell Growth Differ* **5:** 55–60, 1994). The present studies were performed to examine changes in drug sensitivity that accompanied these phenotypic changes. v-*ras*^{H} expression was associated with increased metallothionein-IIa (MT-IIa) mRNA and decreased levels of nonprotein sulfhydryls in NCI-H82*ras*^{H} cells compared with -H82 cells. These changes were accompanied by the development of $CdCl_2$ resistance without any change in cisplatin sensitivity. In contrast, growth of parental NCI-H82 cells in 1 μM tRa resulted in increased MT-IIa mRNA without any change in nonprotein sulfhydryls. In these cells, a 3.3-fold increase in cisplatin IC_{50} was observed. Examination of the action of topoisomerase (topo) poisons revealed that NCI-H82 and -H82*ras*^{H} cells had indistinguishable levels of topo II polypeptides and indistinguishable sensitivities to etoposide, an agent that is often combined with cisplatin clinically. On the other hand, v-*ras*^{H} expression was accompanied by a 2-fold increase in topo I activity and a 1.7-fold decrease in IC_{50} for the topo I-directed agent camptothecin. These changes resulted in 30-fold lower survival of NCI-H82*ras*^{H} cells compared with -H82 cells at camptothecin concentrations as low as 10 nM. In summary, these studies demonstrate that chronic tRA treatment is accompanied by decreased cisplatin sensitivity in NCI-H82 human SCLC cells. In contrast, v-*ras*^{H} expression is not associated with any change in cisplatin or etoposide sensitivity, but is accompanied by increased camptothecin sensitivity.

Key words: ras oncogene; topoisomerases; cisplatin; retinoids; lung cancer

Mutated *ras* oncogenes appear to play an important role in the pathogenesis of human neoplasms (reviewed in Refs. 1 and 2). In NSCLC§, for example, mutated *ras* oncogenes have been detected in tumor-derived cell lines [3, 4] and in as many as 30% of clinical specimens [2, 5, 6]. Subsequent studies have indicated that expression of mutated *ras* oncogenes has an adverse impact on survival of NSCLC patients [6–8]. The etiology of this adverse effect remains unclear.

A number of observations suggest that expression of mutated *ras* oncogenes affects the efficacy of cisplatin, an agent that is widely utilized to treat lung cancer. Murine fibroblasts transformed with the v-*ras*^{H} oncogene contain elevated levels of MT-II mRNA and protein relative to nontransformed cells [9–11]. This *ras*-associated change in MT-II expression is accompanied by 8- to 12-fold increases in the IC_{50} for cisplatin in some cell types [11–13], although much smaller changes have been observed in other cells [14–16].

Expression of *ras* oncogenes also causes changes that could affect sensitivity to etoposide, a topo II-directed agent that is often combined with cisplatin to treat NSCLC [17, 18]. In 3T3 murine fibroblasts, expression of C-H-*ras* enhances expression from the promoter of the *MDR*1 gene [19], a gene that has been implicated in etoposide resistance. On the other hand, transfection of murine fibroblasts with mutated *ras* oncogenes also results in increased levels of topo IIα [20, 21], a change that would be expected to increase etoposide sensitivity (reviewed in Refs. 22–24).

Finally, transfection of rat fibroblasts with the c-*ras*^{H} oncogene has been reported to cause a decrease in activity of topo I [25], a change that would be expected to decrease sensitivity to camptothecin analogues (reviewed in Refs. 22, 26, and 27). In contrast, transformation of murine fibroblasts with Moloney or Kirsten sarcoma viruses or with simian virus 40 has been associated with increased topo I activity [28].

The present studies were undertaken to examine the effect of v-*ras*^{H} expression on the sensitivity of NCI-H82 human lung cancer cells to cisplatin, etoposide and CPT. The parental NCI-H82 cells display phenotypic characteristics commonly seen in SCLC cells including growth as floating aggregates in tissue culture, expression of neuroendocrine markers, and retention of exquisite sensitivity to the cytotoxic effects of the ornithine decarboxylase inhibitor difluoromethylornithine [29]. Introduction of the v-*ras*^{H} oncogene results in a cell line (NCI-H82*ras*^{H}) that displays multiple NSCLC characteristics including growth as a monolayer in tissue culture, decreased expression of neuroendocrine markers,

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§ Abbreviations: CPT, camptothecin; MT-II, metallothionein II; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; PMSF, α-phenylmethylsulfonyl fluoride; SCLC, small cell lung cancer; TCA, trichloroacetic acid; topo, topoisomerase; and tRA, all-*trans*-retinoic acid.

increased expression of intermediate filament proteins, and acquisition of resistance to difluoromethylornithine [29]. The drug sensitivities of this pair of cell lines were examined to assess the role of mutated *ras*^{H} in modulating the responsiveness of human lung cancer cells to chemotherapy.

MATERIALS AND METHODS

Materials

Escherichia coli containing an insert for human genomic MT-IIa [30], the MT-II isoform most often associated with cisplatin resistance [31], were purchased from the American Type Culture Collection (Rockville, MD). Etoposide was a gift from Bristol-Myers Squibb (Wallingford, CT). $CdCl_2$, CPT, glutathione, bromobimane, tRA, and MTT were obtained from the Sigma Chemical Co. (St. Louis, MO). CPT was prepared as a 10 mM stock in DMSO and stored at –20° until used. Multiple aliquots of a 1 mM stock of tRA in ethanol were stored at –20° in foil-wrapped containers until used.

Tissue culture

Retrovirus-mediated insertion of v-*ras*^{H} was performed as described [29]. The introduction of v-*ras*^{H} by this technique avoids position effects that can be observed when foreign genes are inserted by plasmid-mediated transfection and subsequent selection. The resulting NCI-H82*ras*^{H} cells, like the parental -H82 cells, were propagated routinely in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mM glutamine.

The retinoic acid receptor status of these cell lines has been reported previously [32, 33]. Prior to tRA treatment, cells were adapted to RPMI 1640 medium containing 10 nM hydrocortisone, 15 μg/mL insulin, 100 μg/mL transferrin, 10 nM 17β-estradiol, and 30 nM selenium (RPMI-HITES) for 2 weeks. Cells were then propagated in RPMI-HITES in the absence or presence of 1 μM tRA for at least 2 more weeks prior to the initiation of any experiments.

Colony-forming assays

Drug sensitivity of NCI-H82 and -H82*ras*^{H} cells was assessed simultaneously using a continuous exposure protocol. Cell lines harvested in log phase were triturated to single cell suspensions by passage up and down a pipette (generally 50 passages for NCI-H82 and 20 passages for -H82*ras*^{H} cells). Aliquots containing $10^3 - 10^4$ cells were plated in 1 mL of 0.3% (w/v) agar containing various concentrations of $CdCl_2$, cisplatin, etoposide, or CPT in the medium described by Pike and Robinson [34]. Control plates received an equivalent volume of the drug diluent DMSO, which never exceeded 0.1% (v/v). After plates were incubated for 10–14 days at 37° in an atmosphere containing 7.5% (v:v) CO_2, colonies containing >32 cells were counted on an inverted phase contrast microscope. This continuous exposure schedule was chosen to mimic prolonged exposure protocols of topo-directed agents that are being tested clinically [35, 36]. Continuous exposure would also be expected to minimize any effect of differences in cell cycle distribution that arise as a consequence of v-*ras*^{H}-induced shortening of the population doubling time from ~24 hr in NCI-H82 cells to ~18 hr in -H82*ras*^{H} cells [29].

MTT dye reduction assay

Because NCI-H82 cells do not form colonies under serum-free conditions, the effect of tRA on cisplatin sensitivity was assessed using MTT assays. Cells were gently triturated 10 times in a 5-mL pipette to break up large clumps. Triplicate 0.5-mL aliquots containing 12,500 cells in RPMI 1640-HITES ± 1 μM tRA were placed in 24-well dishes containing the indicated final concentration of cisplatin. After a 6-day incubation, MTT (167 μg in 100 μL RPMI-HITES) was added to each well for the final 12 hr. Cells were then washed in serum-free RPMI 1640 and extracted with DMSO containing 1% (w/v) thiodiglycol. The absorbance of the reduced formazan at 540 nm was determined spectrophotometrically as previously described [37].

Northern blotting

Inserts containing cDNA sequences complementary to MT-IIa [30], phosphoglyceraldehyde dehydrogenase [37], c-*myc* or v-*ras*^{H} [33] were excised from plasmids with appropriate restriction enzymes, purified by agarose gel electrophoresis, electroeluted, and labeled by the random primer method. Aliquots of oligo-dT-selected RNA (20 μg) were subjected to electrophoresis, transferred to nylon, and probed as previously described [33, 37].

Western blotting

Log phase cells were washed once in serum-free medium and solubilized by sonication in 6 M guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.5 at 21°), 10 mM EDTA, 1% (v/v) β-mercaptoethanol, and 1 mM freshly added PMSF. Samples were prepared for SDS–PAGE as previously described [38, 39]. Western blotting was performed using the following antibodies: rabbit serum IID, which recognizes the 170 kDa isoform of topo II [38], affinity-purified rabbit anti-human topo I ([40], provided by Dr. Leroy Liu, Robert Wood Johnson Medical School, Piscataway, NJ), and murine monoclonal anti-histone H1 (provided by Dr. James Sorace, Veterans Administration Hospital, Baltimore, MD). The resulting autoradiographs were scanned as recently described [38, 39].

Topo I activity assay

Nuclear extracts were prepared from logarithmically growing cells and assayed for topo I activity as previously described [37, 39]. In brief, NCI-H82 or trypsinized -H82*ras*^{H} cells were sedimented at 200 *g* for 10 min. All further steps were performed at 4° unless indicated. Cells were washed twice in RPMI 1640 containing 10 mM HEPES (pH 7.4); incubated for 20 min in nuclear isolation buffer consisting of 10 mM NaCl, 10 mM Tris-HCl (pH 7.4 at 4°), 3 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 100 U/mL aprotinin, and 1 mM freshly added PMSF; and lysed with 25 strokes of a tight-fitting Dounce homogenizer. Nuclei were sedimented at 800 *g* for 10 min; washed once with fresh nuclear isolation buffer; and incubated for 15 min with extraction buffer consisting of 0.6 M KCl, 100 mM sodium phosphate (pH 7.4 at 4°), 1 mM EDTA, 0.1 mM dithiothreitol, and 1 mM freshly added PMSF. One-half volume of 13% (w/v) PEG8000 polyethylene glycol was then added. After an additional 15-min incubation, sam-

ples were sedimented at 16,000 *g* for 5 min. The supernatant was saved for determination of protein content [41] and topo I activity.

To assay topo I activity, 2-μL aliquots containing the indicated amount of protein were added to samples (20 μL final volume) containing 450 ng of supercoiled plasmid 067 [37], 100 mM NaCl, 50 mM Tris-HCl (pH 7.5 at room temperature), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 50 μg/mL bovine serum albumin. After a 30-min incubation at 37°, the reaction was terminated by adding 1 μL of 10% (w/v) SDS and 1 μL of 10 mg/mL proteinase K. Samples were incubated at 37° for an additional 15 min and subjected to agarose gel electrophoresis in the presence of chloroquine as previously described [37].

Estimation of nonprotein sulfhydryl groups

Logarithmically growing cells were washed once with ice-cold serum-free RPMI 1640 medium, extracted for 15 min at 4° with nitrogen-sparged 5% (w/v) TCA, and sedimented at 1600 *g* for 15 min. The supernatant was assayed for content of nonprotein sulfhydryl groups using bromobimane [42]. In brief, 400-μL aliquots were neutralized with 800 μL of 0.4 M Tris-HCl (pH 8.9) containing 15 μM bromobimane. After a 30-min incubation at 21°, samples were examined in a Farrand Mark I fluorometer using an excitation wavelength of 366 nm and an emission wavelength of 477 nm. Samples containing 0–60 nmol of glutathione in 400 μL of 5% TCA were utilized to construct a standard curve. Protein in the cell pellet was solubilized by heating at 70° for 30 min in 0.5 M NaOH, neutralized, and assayed by the bicinchoninic acid method [43].

RESULTS

Effect of v-ras^H expression on cisplatin sensitivity

The following experiments were undertaken to determine whether the v-*ras*^{H}-induced change from SCLC to NSCLC phenotype in the NCI-H82 cell line [29] was accompanied by changes in drug sensitivity. Northern blotting confirmed that the NCI-H82 and -H82*ras*^{H} cell lines both expressed high levels of the c-*myc* oncogene (Fig. 1A, lanes 1 and 2). As expected, the v-*ras*^{H} oncogene was expressed only in the v-*ras*^{H} transfected cell line (Fig. 1B, lane 2). The NCI-H82*ras*^{H} cells also contained higher levels of MT-II mRNA (Fig. 1C, cf. lanes 1 and 2). Consistent with this result, MTT dye reduction assays (not shown) and clonogenic assays (Fig. 2A) revealed that the NCI-H82*ras*^{H} cells were less sensitive to the metallothionein-detoxified heavy metal Cd^{2+}. In four independent experiments, a 2.7 ± 1.0-fold (mean ± SD) increase in $CdCl_2$ IC_{50} was observed.

When cisplatin cytotoxicity was assessed by MTT and clonogenic assays, the increased MT-II expression in the NCI-H82-*ras*^{H} cells was not accompanied by major changes in cisplatin sensitivity (Fig. 2B). In twelve separate assays utilizing cell lines obtained from two independent v-*ras*^{H} transfections, the IC_{50} for cisplatin in the NCI-H82*ras*^{H} cells was an average of 1.3 (SD ± 0.8) times the IC_{50} determined simultaneously in parental NCI-H82 cells. This change in cisplatin sensitivity was much smaller than the change expected based on results observed when murine fibroblast lines were transfected with v-*ras*^{H} [10–13].

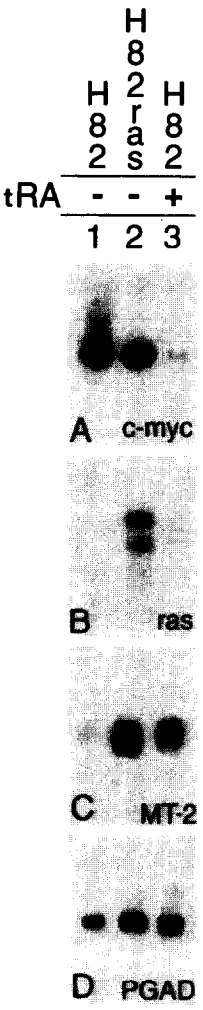

Fig. 1. Effect of v-*ras*^{H} or tRA on expression of MT-2. Oligo-(dT)-selected RNA (20 μg) from NCI-H82 cells grown in the absence of tRA (lane 1), -H82*ras*^{H} cells grown in the absence of tRA (lane 2) or -H82 cells grown in the presence of 1 μM tRA (lane 3) was subjected to agarose gel electrophoresis in the presence of formaldehyde, transferred to nylon, and probed with cDNA that was complementary to c-*myc* (A), v-*ras*^{H} (B), MT-2 (C), or the housekeeping gene phosphoglyceraldehyde dehydrogenase (D). Results are representative of 2 independent experiments.

Effect of tRA on cisplatin sensitivity

Treatment of NCI-H82 cells with tRA also resulted in increased MT-II mRNA (Fig. 1C, lane 3). In this case, however, the increase in MT-II expression was accompanied by a 3.3-fold increase (SD ± 0.4-fold, N = 3) in cisplatin IC_{50} (Fig. 2C, squares). In an attempt to explain the apparent discrepancy between results obtained after v-*ras*^{H} transfection and tRA treatment, levels of nonprotein sulfhydryl groups were assayed in lysates from these cells. As shown in Fig. 3, nonprotein sulfhydryl groups were diminished by 40% in NCI-H82*ras*^{H} cells compared with parental cells, but a similar decrease was not observed in tRA-treated cells. In view of the role of glutathione in cisplatin resistance [44, 45], these observations suggest that a decrease in glutathione content might have offset the effect of the increased MT-II ex-

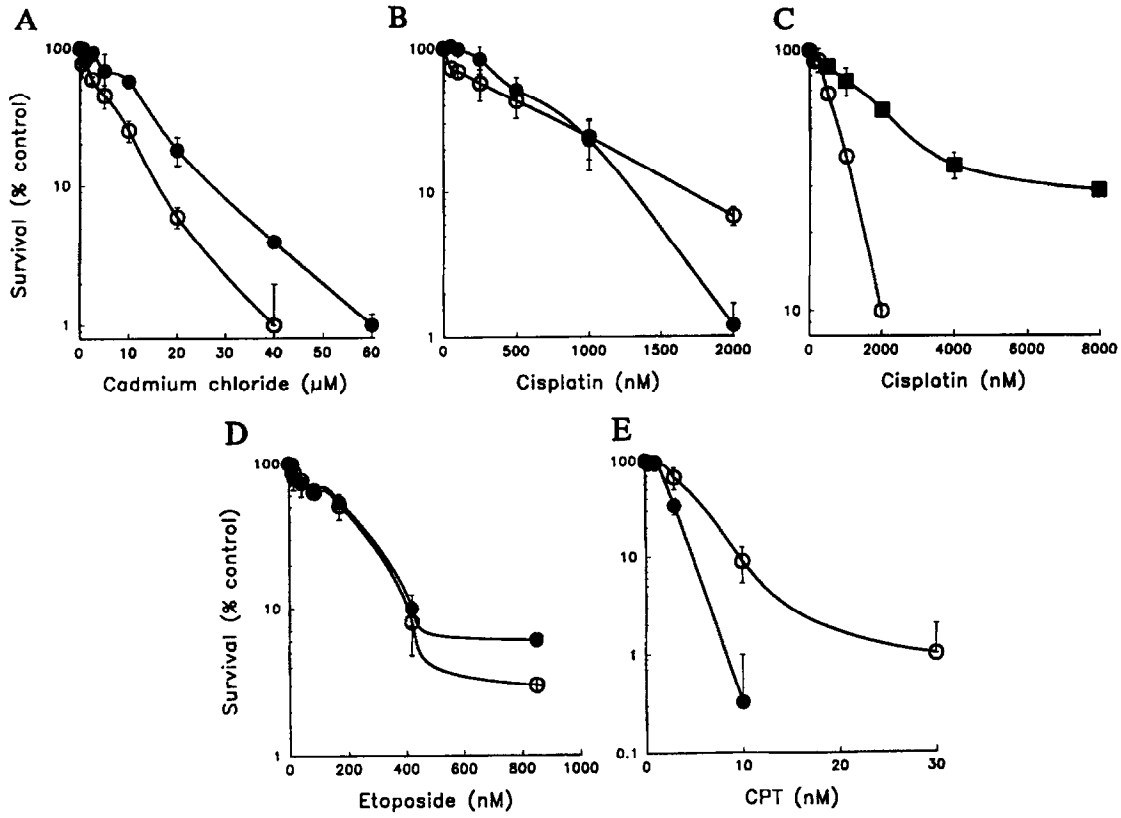

Fig. 2. Response of cell lines to drug treatment. Sensitivities of NCI-H82 (○) and -H82*ras*^{H} cells (●) to cadmium (A), cisplatin (B), etoposide (D), or CPT (E) were determined using clonogenic assays. Error bars, ±1 SD. In these assays, control samples generally had 200–300 colonies. To assess the effect of tRA on cisplatin sensitivity (C), NCI-H82 cells grown in RPMI-HITES in the absence of tRA (○) or presence of 1 μM tRA (■) were assayed for sensitivity using an MTT assay. All results are representative of ≥ 3 independent experiments.

pression in the v-*ras*^{H}-transfected cells but not in tRA-treated cells.

Effect of v-ras^{H} *expression on etoposide sensitivity*

To determine whether v-*ras*^{H} expression altered the sensitivity to other agents utilized to treat SCLC, the topo II-directed agent etoposide was evaluated. Examination of the NCI-H82 and -H82*ras*^{H} cell lines by western blotting revealed that the amount of topo IIα polypeptide was indistinguishable (Fig. 4C, lanes 1 and 4). Similar results were obtained using an antiserum that recognized both topo II isoforms (data not shown). Consistent with this result, clonogenic assays failed to reveal any difference in sensitivities of the NCI-H82 and -H82*ras*^{H} cell lines to etoposide (Fig. 2D).

*Increased sensitivity of NCI-H82*ras^{H} *to camptothecin*

In addition to cisplatin and etoposide, the topo I-directed CPT analogues have shown clinical activity in SCLC and NSCLC (reviewed in Refs. 26 and 27). In view of these results, the cytotoxicity of the prototypic agent CPT was examined in the NCI-H82 and -H82*ras*^{H} cells. NCI-H82*ras*^{H} cells displayed decreased survival at all cytotoxic drug concentrations when compared with simultaneously exposed -H82 cells (Fig. 2E). At 10 nM CPT, survival of NCI-H82*ras*^{H} cells was 1/30th that of NCI-H82 cells. Expressed in different terms, the NCI-H82*ras*^{H} cells exhibited an IC_{50} that was 0.6 times the IC_{50} simultaneously determined for parental -H82 cells (SD ± 0.2, N = 4).

Experiments undertaken to assess the mechanism of this enhanced CPT sensitivity revealed that topo I activ-

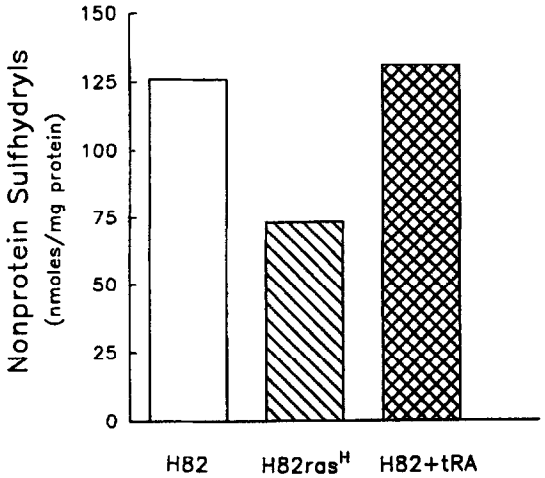

Fig. 3. Effect of v-*ras*^{H} transfection and tRA treatment on levels of nonprotein sulfhydryl groups in NCI-H82 cells. Cells grown in RPMI-HITES medium in the absence (left and middle bars) or presence of 1 μM tRA (right bar) were assayed as described in Materials and Methods. Results are representative of 3 independent experiments.

ity was 2-fold higher in nuclear extracts from NCI-H82*ras*^{H} cells compared with -H82 cells (cf. Fig. 5A, lane 4, and Fig. 5B, lane 5). This increase was similar in magnitude to the increase in topo I activity observed in murine fibroblasts transformed by a variety of viruses [28] but appears to be slightly out of proportion to the 30% increase in immunoreactive topo I polypeptide observed on western blots (Fig. 4B, cf. lanes 1 and 4).

DISCUSSION

Differences in response of SCLC and NSCLC to the same therapeutic regimen are striking. Up to 80% of tumors in patients with limited-stage SCLC respond to therapy with cisplatin and etoposide [46, 47]. In contrast, the same regimen has a 20–30% response rate in patients with NSCLC [18, 48, 49]. In an attempt to investigate the causes of the decreased response rate in NSCLC, we examined a unique series of human lung cancer cell lines derived from NCI-H82 SCLC cells. In this model system, expression of mutated v-*ras*^{H} is accompanied by profound biochemical and morphological changes that result in cells with NSCLC phenotype [29]. Despite these phenotypic changes, we observed that expression of v-*ras*^{H} was accompanied by minimal changes in cisplatin and etoposide sensitivity. Thus, the phenotypic change from SCLC to NSCLC *per se* is not sufficient to account for the decreased sensitivity of NSCLC to these agents.

Although the present results might be construed as a negative study, several findings should be noted. First, introduction of v-*ras*^{H} in the NCI-H82 SCLC line was accompanied by increased MT-II expression (Fig. 1C) and development of resistance to cadmium (Fig. 2A) without a major change in cisplatin sensitivity (Fig. 2B). At first glance these results appear to contradict previous observations that transfection of v-*ras*^{H} into murine fibroblasts results in an 8- to 12-fold change in cisplatin IC_{50} [11, 13]. Several factors including differences in the cell type and species from which the cell lines originated might contribute to these disparate results, but the decreased levels of nonprotein sulfhydryl groups in the NCI-H82*ras*^{H} cells compared with -H82 cells (Fig. 3) very likely play a major role.

In contrast to the v-*ras*^{H}-associated changes, chronic treatment of NCI-H82 cells with tRA resulted in increased MT-II expression (Fig. 1C) in the absence of changes in nonprotein sulfhydryl groups (Fig. 3). As a consequence, tRA treatment of NCI-H82 cells was associated with decreased cisplatin sensitivity (Fig. 2C). This result is in sharp contrast to recent reports indicating that tRA increases the cisplatin sensitivity of squamous cell carcinoma [50] and some ovarian carcinoma lines [51]. These disparate observations might reflect differences in cell types or differences in the schedule of administration of tRA and cisplatin. In view of current interest in retinoids as agents to prevent second malignancies after treatment for tumors of the upper aerodigestive tract [52], the effect of tRA on cisplatin sensitivity of a variety of cell types under a variety of conditions clearly requires further investigation.

Likewise, the effect of v-*ras*^{H} on sensitivity to topo-directed agents requires further study. Although studies in murine fibroblasts raised the possibility that expression of mutated *ras* oncogenes might be accompanied by increased topo IIα polypeptide content (see beginning of paper), the NCI-H82 and -H82*ras*^{H} lines had indistinguishable topo IIα polypeptide levels (Fig. 4C) and indistinguishable sensitivities to the topo II-directed agent etoposide (Fig. 2D). In contrast, introduction of v-*ras*^{H} was associated with increased CPT sensitivity (Fig. 2E). This altered CPT sensitivity most likely reflects the v-*ras*^{H}-induced increase in topo I activity (Fig. 5), although a contribution of the v-*ras*^{H}-associated change in cellular growth kinetics [29] also cannot be excluded. Collectively, these changes resulted in 30-fold lower survival of NCI-H82*ras*^{H} cells compared with -H82 cells at

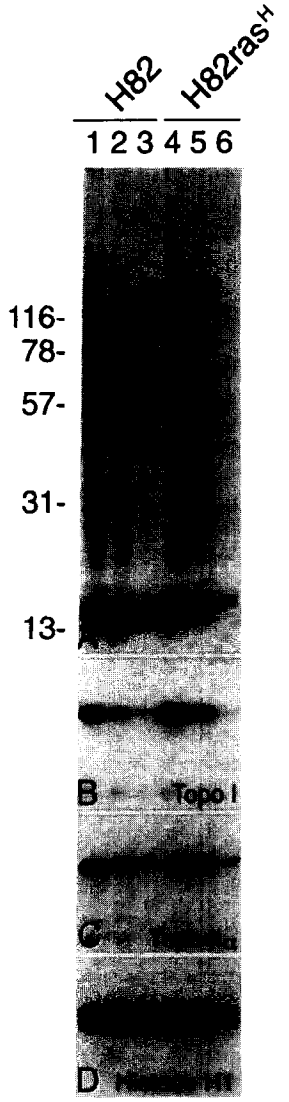

Fig. 4. Western blot of NCI-H82 and -H82*ras*^{H} for relative topoisomerase levels. Samples containing 30 μg (lanes 1 and 4), 15 μg (lanes 2 and 5), and 7.5 μg (lanes 3 and 6) of total protein from NCI-H82 cells (lanes 1–3) or -H82*ras*^{H} cells (lanes 4–6) were subjected to SDS–PAGE followed by staining with Coomassie blue (A) or transfer to nitrocellulose and reaction with antibodies that recognize topo I (B) or topo II (C). To confirm that samples were loaded with protein from an equivalent number of cells, the blots were reprobed with a monoclonal antibody that recognizes histone H1 (D), a polypeptide whose level is closely linked to cellular DNA content. Numbers on the left indicate the size of molecular weight standards in kilodaltons. Results from this experiment are representative of 3 independent experiments.

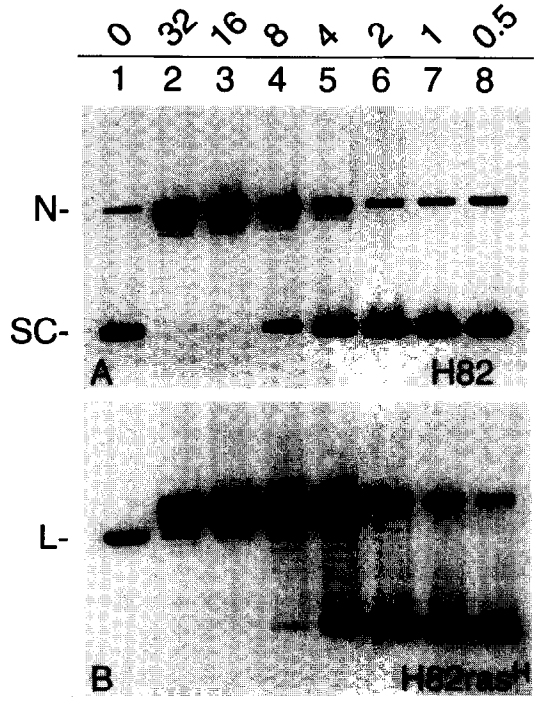

Fig. 5. Titration assay for topo I activity. Nuclear extracts were prepared simultaneously from NCI-H82 cells (A) and -H82*ras*^{H} cells (B). Samples containing the indicated amounts of extract protein (in ng) were assayed for their ability to relax 450 ng of supercoiled plasmid (SC, lane 1). Since 8 ng of nuclear extract from NCI-H82 cells but only 4 ng of nuclear extract from -H82*ras*^{H} cells is required to relax half of the substrate (cf. lane 4 in A and lane 5 in B), -H82*ras*^{H} extracts contain approximately twice as much topo I activity as -H82 extracts. Lane 1 contains starting material consisting of supercoiled (SC) and nicked (N) plasmid (panel A) or linearized (L) plasmid (panel B). Results are representative of 2 independent assays.

CPT concentrations as low as 10 nM (Fig. 2E), concentrations that are readily surpassed in clinical trials (reviewed in Ref. 26). When expressed in terms of IC_{50} values, introduction of v-*ras*^{H} was accompanied by a 1.7-fold decrease in CPT IC_{50}. Even though this IC_{50} decrease sounds small, it is important to realize that an 8-fold increase in CPT IC_{50} in murine P388 leukemia cells is associated with conversion of a curable neoplasm into one that is totally unresponsive to CPT *in vivo* [53]. Conversely, a decrease of topo I activity as small as 2-fold has been associated with a decrease in topotecan response rate from 3/9 to 0/10 in human rhabdomyosarcoma xenografts *in vivo* [39]. These considerations suggest that the IC_{50} decrease observed in the present study (Fig. 2E) should not be immediately dismissed. Instead, the present observations raise the possibility that the NSCLC patients responding to the CPT analogs irinotecan or topotecan in clinical trials may be the subset of patients whose tumors contain mutated *ras* oncogenes [2, 6]. Further studies are required to assess this possibility.

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REFERENCES

1. Barbacid M, *ras* Genes. *Annu Rev Biochem* **56:** 779–827, 1987.
2. Bos JL, *ras* Oncogenes in human cancer: A review. *Cancer Res* **49:** 4682–4689, 1989.
3. Der CJ and Cooper GM, Altered gene products are associated with activation of cellular *ras*^{k} genes in human lung and colon carcinomas. *Cell* **32:** 201–208, 1983.
4. Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G and Barbacid M, Malignant activation of a K-*ras* oncogene in lung carcinoma but not in normal tissue of the same patient. *Science* **223:** 661–664, 1984.
5. Kurzrock R, Gallick GE and Gutterman JU, Differential expression of p21^{ras} gene products among histological subtypes of fresh primary human lung tumors. *Cancer Res* **46:** 1530–1534, 1986.
6. Sugio K, Ishida T, Yokoyama H, Inoue T, Sugimachi K and Sasazuki T, ^{ras} Gene mutations as a prognostic marker in adenocarcinoma of the human lung without lymph node metastasis. *Cancer Res* **52:** 2903–2906, 1992.
7. Slebos RJC, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJLM, Wagenaar SS, Vanderschueren RGJRA, van Zandwijk N, Mooi WJ, Bos JL and Rodenhuis S, K-*ras* oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* **323:** 561–565, 1990.
8. Miyamoto H, Harada M, Isobe H, Akita HD, Haneda H, Yamaguchi E, Kuzumaki N and Kawakami Y, Prognostic value of nuclear DNA content and expression of the *ras* oncogene product in lung cancer. *Cancer Res* **51:** 6346–6350, 1991.
9. Schmidt CJ and Hamer DH, Cell specificity and an effect of *ras* on human metallothionein gene expression. *Proc Natl Acad Sci USA* **83:** 3346–3350, 1986.
10. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH and Lazo JS, Overexpression of metallothionein confers resistance to anticancer drugs. *Science* **241:** 1813–1815, 1988.
11. Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A and Howell SB, Expression of the c-Ha-*ras* oncogene in mouse NIH 3T3 cells induces resistance to cisplatin. *Cancer Res* **5:** 5903–5909, 1991.
12. Sklar MD, Increased resistance to *cis*- diamminedichloroplatinum (II) in NIH 3T3 cells transformed by *ras* oncogenes. *Cancer Res* **48:** 793–797, 1988.
13. Peters GJ, Wets M, Keepers YPAM, Oskam R, van Ark-Otte J, Noordhuis P, Smid K and Pinedo HM, Transformation of mouse fibroblasts with the oncogenes H-*ras* or *trk* is associated with pronounced changes in drug sensitivity and metabolism. *Int J Cancer* **54:** 450–455, 1993.
14. Schilder RJ, Hall L, Monks A, Handel LM, Fornace AJ Jr, Ozols RF, Fojo AT and Hamilton TC, Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. *Int J Cancer* **45:** 416–422, 1990.
15. Niimi S, Nakagawa K, Yokota J, Tsunokawa Y, Nishio K, Terashima Y, Shibuya M, Terada M and Saijo N, Resistance to anticancer drugs in NIH3T3 cells transfected with c-*myc* and/or c-H-*ras* genes. *Br J Cancer* **63:** 237–241, 1991.
16. Morton KA, Jones BJ, Sohn M-H, Datz FL and Lynch RE, Enrichment for metallothionein does not confer resistance to cisplatin in transfected NIH/3T3 cells. *J Pharmacol Exp Ther* **267:** 697–702, 1993.
17. Ruckdeschel JC, The role of standard-dose etoposide in the management of non-small cell lung cancer. *Semin Oncol* **19** (Suppl 13): 39–44, 1992.

18. Shaw GL, Gazdar AF, Phelps R, Linnoila RI, Ihde DC, Johnson BE, Oie HK, Pass HI, Steinberg SM, Ghosh BC, Walsh TE, Nesbitt JC, Cotelingam JD, Minna JD and Mulshine JL, Individualized chemotherapy for patients with non-small cell lung cancer determined by prospective identification of neuroendocrine markers and *in vitro* drug sensitivity testing. *Cancer Res* **53:** 5181–5187, 1993.
19. Chin K-V, Ueda K, Pastan I and Gottesman MM, Modulation of activity of the promoter of the human *MDR*1 gene by Ras and p53. *Science* **255:** 459–462, 1992.
20. Woessner RD, Chung TDY, Hofmann GA, Mattern MR, Mirabelli CK, Drake FH and Johnson RK, Differences between normal and *ras*-transformed NIH-3T3 cells in expression of the 170kD and 180kD forms of topoisomerase II. *Cancer Res* **50:** 2901–2908, 1990.
21. Cunningham JM, Francis GE, Holland MJ, Pirollo KF and Chang EH, Aberrant DNA topoisomerase II activity, radioresistance and inherited susceptibility to cancer. *Br J Cancer* **63:** 29–36, 1991.
22. Liu LF and D'Arpa P, Topoisomerase-targeting antitumor drugs: Mechanisms of cytotoxicity and resistance. In: *Important Advances in Oncology 1992* (Eds. DeVita VT Jr, Hellman S and Rosenberg SA), pp. 79–89. J. B. Lippincott, Philadelphia, PA, 1992.
23. Alton PA and Harris AL, The role of DNA topoisomerase II in drug resistance. *Br J Haematol* **85:** 241–245, 1993.
24. Giaccone G, Small cell lung cancer and topoisomerases. *Anticancer Res* **14:** 269–276, 1994.
25. Basnak'yan AG, Topol' LZ, Kirsanova ID, Votrin II and Kiselev FL, Activity of topoisomerase I and endonucleases in cells transformed by ras oncogene. *Mol Biol USSR* **23:** 588–594, 1989.
26. Slichenmyer WJ, Rowinsky EK, Donehower RC and Kaufmann SH, The current status of camptothecin analogues as antitumor agents. *J Natl Cancer Inst* **85:** 271–291, 1993.
27. Potmesil M, Camptothecins: From bench research to hospital wards. *Cancer Res* **54:** 1431–1439, 1994.
28. Crespi MD, Mladovan AG and Baldi A, Increment of DNA topoisomerases in chemically and virally transformed cells. *Exp Cell Res* **175,** 206–215, 1988.
29. Mabry M, Nakagawa T, Nelkin BD, McDowell E, Gesell M, Eggleston JC, Casero RA Jr and Baylin SB, v-Ha-*ras* oncogene insertion: A model for tumor progression of human small cell lung cancer. *Proc Natl Acad Sci USA* **85:** 6523–6527, 1988.
30. Richards RI, Heguy A, and Karin M, Structural and functional analysis of the human metallothionein-1_A gene: Differential induction by metal ions and glucocorticoids. *Cell* **37:** 263–272, 1984.
31. Yang Y-Y, Woo ES, Reese CE, Bahnson RR, Saijo N and Lazo JS, Human metallothionein isoform gene expression in cisplatin-sensitive and resistance cells. *Mol Pharmacol* **45:** 453–460, 1994.
32. Geradts J, Chen JY, Russell EK, Yankaskas JR, Nieves L and Minna JD, Human lung cancer cell lines exhibit resistance to retinoic acid treatment. *Cell Growth Differ* **4:** 799–809, 1993.
33. Kalemkerian GP, Jasti RK, Celano P, Nelkin BD and Mabry M, All-*trans*-retinoic acid alters *myc* gene expression and inhibits *in vitro* progression in small cell lung cancer. *Cell Growth Differ* **5:** 55–60, 1994.
34. Pike BL and Robinson WA, Human bone marrow colony growth in agar-gel. *J Cell Physiol* **76:** 77–84, 1970.
35. DeVore R, Hainsworth J, Greco FA, Hande K and Johnson D, Chronic oral etoposide in the treatment of lung cancer. *Semin Oncol* **19**(Suppl 14): 28–35, 1992.
36. Hochster H, Liebes L, Speyer J, Sorich J, Taubes B, Oratz R, Wernz J, Chachoua A, Raphael B, Vinci RZ and Blum RH, Phase I trial of low-dose continuous topotecan infusion in patients with cancer: An active and well-tolerated regimen. *J Clin Oncol* **12:** 553–559, 1994.
37. Hendricks CB, Rowinsky EK, Grochow LB, Donehower RC and Kaufmann SH, Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res* **52:** 2268–2278, 1992.
38. Kaufmann SH, McLaughlin SJ, Kastan MB, Liu LF, Karp JE and Burke PJ, Topoisomerase II levels during granulocytic maturation *in vitro* and *in vivo*. *Cancer Res* **51:** 3534–3543, 1991.
39. Friedman HS, Dolan ME, Kaufmann SH, Colvin OM, Griffith OW, Moschel RC, Schold SC, Bigner DD and Ali-Osman F, Elevated DNA polymerase α, DNA polymerase β, and DNA topoisomerase II in a melphalan-resistant rhabdomyosarcoma xenograft that is cross-resistant to nitrosoureas and topotecan. *Cancer Res* **54:** 3487–3493, 1994.
40. Hsiang Y-H and Liu LF, Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res* **48:** 1722–1726, 1988.
41. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
42. Kosower NS, Kosower EM, Newton GL and Ranney HM, Bimane fluorescent labels: Labeling of normal human red cells under physiological conditions. *Proc Natl Acad Sci USA* **76:** 3382–3386, 1979.
43. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
44. Mistry P, Kelland LR, Abel G, Sidhar S and Harrap KR, The relationships between glutathione, glutathione-*S*-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br J Cancer* **64:** 215–220, 1991.
45. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC and Anderson ME, High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci USA* **89:** 3070–3074, 1992.
46. Evans WK, Shepherd FA, Feld R, Osoba D, Dang P and Deboer G, VP-16 and cisplatin as first-line therapy for small-cell lung cancer. *J Clin Oncol* **3:** 1471–1477, 1985.
47. Aisner J, Treatment of limited-disease small cell lung cancer. *Semin Oncol* **19** (Suppl 13): 51–58, 1992.
48. Ruckdeschel JC, Finkelstein DM, Ettinger DS, Creech RH, Mason BA, Joss RA and Wogl S, A randomized trial of the four most active regimens for metastatic non-small-cell lung cancer. *J Clin Oncol* **4:** 14–22, 1986.
49. Crino L, Tonato M, Darwish S, Meacci ML, Corgna E, Dicostanzo F, Buzzi F, Fornari G, Santi E, Ballatori E, Santucci C and Davis S, A randomized trial of three cisplatin-containing regimens in advanced non-small-cell lung cancer (NSCLC): A study of the Umbrian Lung Cancer Group. *Cancer Chemother Pharmacol* **26:** 52–56, 1990.
50. Sacks PG and Harris D, Retinoic acid induced growth inhibition in squamous cell carcinoma permits continuing DNA synthesis: A rationale for combination therapy. *Proc Am Assoc Cancer Res* **34:** 430, 1993.
51. Nehme A, Julia AM, Jozan S, Bugat R and Canal P, Modulation of cisplatin cytotoxicity by human recombinant interferon gamma and all *trans*-retinoic acid in four human ovarian cell lines. *Proc Am Assoc Cancer Res* **34:** 295, 1993.
52. Lippman SM and Hong WK, Retinoid chemoprevention of upper aerodigestive tract carcinogenesis. In: *Important Advances in Oncology 1992* (Eds. DeVita VT Jr, Hellman S and Rosenberg SA), pp. 93–109. J. B. Lippincott, Philadelphia, PA, 1992.
53. Eng WK, McCabe FL, Tan KB, Mattern MR, Hofmann GA, Woessner RD, Hertzberg RP and Johnson RK, Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. *Mol Pharmacol* **38:** 471–480, 1990.