Transformation of Rat Ovarian Epithelial and Rat-1 Fibroblast Cell Lines by RAST24 Does Not Influence Cisplatin Sensitivity

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ABSTRACT

Recent reports suggest that expression of an activated c-Ha-ras oncogene is associated with cisplatin resistance in NIH-3T3 fibroblasts. To investigate the generality of these observations, cisplatin cytotoxicity was determined in a series of clonal Rat-1 fibroblast and rat ovarian surface epithelial (ROSE) cell lines carrying a zinc-inducible metallothionein-RAST24 fusion gene, MTRAST24. Cisplatin sensitivity in RAS-transformed fibroblast sublines did not differ from parental controls. Induction of mutant RAST24 expression by zinc sulfate did not affect the cisplatin sensitivity of individual cell lines. Expression of mutant p21^ras varied more than 40-fold in these fibroblast sublines. Similarly, there was no difference in cisplatin sensitivity between parental ROSE controls, neomycin phosphotransferase transfected controls, or MTRAST24 transfectants. Finally, the cisplatin sensitivity of RAS-transformed ROSE cells was similar to that of spontaneously transformed ROSE cells. Overall, these observations suggest that there is little relationship between mutant ras expression and cisplatin sensitivity in rat epithelial and fibroblast cell lines.

INTRODUCTION

Resistance to chemotherapy ranks among the most significant problems in the treatment of cancer patients. It has been estimated that up to 90% of all cancer deaths are related to the presence of intrinsic or acquired drug resistance (1). The development of resistance is particularly troubling among patients with diseases which initially respond to chemotherapy. For example, although the majority of advanced stage ovarian cancer patients respond to cisplatin-based chemotherapy, fewer than 20% survive 5–10 years following treatment (2–4). The remaining patients relapse and develop disease which is refractory to available chemotherapy. The eventual emergence of resistance in these patients has led some to suggest that the development of drug resistance is an integral aspect of the biology of this disease (5). More generally, clinical resistance to chemotherapy is consistent with mathematical models in which resistance arises in conjunction with spontaneous mutations occurring during malignant transformation and progression (6–8).

These observations have led some investigators to examine the effect of mutations in genes potentially involved in cellular proliferation and transformation on sensitivity to anticancer drugs. Considerable attention has been focused upon ras oncogenes. The ras protooncogenes function as regulatory G proteins, modulating cell growth by regulating signal transduction at the cell membrane. Point mutations within critical region of ras protooncogenes can contribute to malignant transformation of certain cell lines (9). Expression of mutant RAS genes may be associated with a more aggressive biology for some cancers and premalignant conditions (10–14). Some investigators have observed that transformation of murine NIH-3T3 fibroblasts by mutant c-Ha-ras oncogenes was associated with increased resistance to cisplatin (15, 16). However, contrary results have been reported by other investigators in similar model systems (17).

The present investigations were undertaken to determine if mutant c-Ha-RAS gene expression is more generally related to cisplatin resistance. With the exception of one preliminary report (18), the published experience with mutant RAS and cisplatin resistance is limited to 3T3 cells. The relevance of cisplatin resistance data obtained in 3T3 fibroblasts to other cell lines remains to be established.

MATERIALS AND METHODS

Cell Lines. Rat-1 is an immortalized, nontransformed fibroblast cell line. MR4, MRS, and MR7 are transformed clonal cell lines derived from Rat-1 following transfection of a mutant human H-RAst gene (c-RAST24) under the control of a zinc-inducible murine metallothionein-1 promoter (19, 20). ROSE3 cells are harvested by selective trypsinization of resected rat ovaries. These cells spontaneously transform following multiple passages in vitro (21). Clone 2 and clone 7 are clonal ROSE sublines. A retroviral vector (SVX) was used to insert a MTRAST24 fusion gene and a neomycin phosphotransferase gene into early passage (No. 8), nontransformed clone 2 and clone 7 cells. Clonal cell lines isolated following G418 selection (500 μg/ml; Gibco BRL, Grand Island, NY) were designated SVX. Cells designated neo6 were obtained by transfection of clone 2 and clone 7 cells with a neomycin phosphotransferase gene, followed by G418 selection. G418-resistant clones were isolated and expanded.

Cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4% fetal bovine serum (GIBCO), 0.28 units/ml insulin (Squibb-Novo, Inc., Princeton, NJ), 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.3 mg/ml glutamine. Cells were grown at 37°C in a humidified atmosphere of 5% CO2 in air.

Drugs. The clinical formulation of cisplatin, provided by Bristol-Myers-Squibb Corporation, was used in cytotoxicity experiments.

Cytotoxicity Assays. Cytotoxicity was determined either by tetrazolium reduction (MTT) or SRB assay, as previously described (22–25). For MTT assays, cells (1000–32,000/well) were plated in 96-well plates and allowed to attach, and cisplatin was added at concentrations from 0 to 10 μM. The percentage of viable cells was determined by reduction of MTT, relative to controls, following 72-h continuous cisplatin treatment. In SRB assays, 5 × 103 cells/well were plated and allowed to attach in 96-well plates, followed by continuous cisplatin for 72 h. At least 6 concentrations of cisplatin were tested in each cell line. Cells were then fixed and stained with SRB. Growth over this time interval, relative to controls, was determined by SRB staining, which correlates linearly with cell number (24–26). Results from the MTT and SRB cytotoxicity assays are highly correlated (24, 26). Selection of the MTT assay for the Rat-1 experiments and of the SRB assay for ROSE experiments was arbitrary. All cytotoxicity data shown are the means of at least 3 independent experiments.

Clonogenic Assay. The capacity of cell lines for substrate-independent growth was determined by soft-agarose clonogenic assay, as previously described (27). Briefly, 50,000 cells/ml of 0.3% agarose (ν/ν; in maintenance medium) were plated over chilled (4°C) 0.6% agarose (ν/ν, in medium) feeder layers. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2.
in air for 14 days. Colonies (minimum diameter, 40 μm) were counted at 7 days and recounted at 14 days (same plates) using an Artek Omnic 3600 image analysis system (Chantilly, VA).

**Metabolic Labeling of Cells and Immunoprecipitation.** Expression of p21^{Hs-RAS} was quantitated by immunoprecipitation, as previously described (28). Briefly, 1 × 10^6 cells were seeded in 60-mm tissue culture dishes (Nunc) and allowed to grow to approximately 70% confluence (1–2 days). Cells were labeled by refeeding with methionine-free Dulbecco’s modified Eagle’s medium (Sigma) containing 7.5% dialyzed fetal calf serum, 100 μCi/ml [35S]-methionine (1078 Ci/mmol; New England Nuclear), and in some cases 75 or 100 μM ZnSO4. Labeling was carried out at 37°C for 24 h. Cell lysates were prepared, and p21^{Hs-RAS} was immunoprecipitated with monoclonal antibody Y13-238 (Oncogene Science, Uniondale, NY; 1:20 dilution) and analyzed by electrophoresis on a 12.5% sodium dodecyl sulfate polyacrylamide gel. The positions of the p21 products of the endogenous and mutant (RAST24) genes were determined relative to molecular weight standards and are indicated in Figs. 1, 4, and 5 by arrows. Relative p21^{Hs-RAS} expression was quantitated within the linear range of the film using an UltraScan XL laser densitometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

**Isolation of Cellular RNA and Northern Blotting.** Isolation of cellular RNA and Northern blotting were performed as previously described (22). Briefly, total RNA (16 μg/lane) was denatured in 50% formamide containing 7.4% formaldehyde and separated by electrophoresis on an agarose gel (1% agarose/2.2 M formaldehyde). RNA was blotted by capillary action onto Magna NT membrane filters (Micron Separations, Westborough, MA) in 10 × standard saline-citrate (1.5 M NaCl-0.15 M sodium citrate; pH 7.0) and hybridized within the linear range of the film using an UltraScan XL laser densitometer.

**RESULTS**

**Rat-1 Fibroblasts and RAS-transformed Sublines.** The RAST24 gene responsible for transformation of the MR sublines contains a point mutation within codon 12 resulting in substitution of valine for glycine. This substitution results in a slightly different electrophoretic mobility for mutant, versus endogenous, p21^{Hs-RAS}, as indicated in Fig. 1. The band for endogenous p21^{Hs-RAS} was faintly visible in all cell lines. In contrast, increasing expression of mutant p21^{Hs-RAS} was clearly seen in the MR4, MR5, and MR7 sublines, in the absence of zinc. Basal levels of mutant RAS p21 were 18-fold higher in MR7 cells than in MR5 cells (not detectible in MR4 cells). Expression was zinc-inducible by 24-fold in MR5 cells and by 2-fold in MR7 cells. Zinc inducibility was also clearly apparent in MR4 cells. Cisplatin did zinc-inducible by 24-fold in MR7 cells (50% inhibitory concentrations all within <2-fold range). In addition, cisplatin sensitivity in all 3 MR sublines was the same in the presence or absence of zinc (100 μM), despite the marked differences in mutant p21^{Hs-RAS} expression apparent under these conditions (Fig. 1).

**ROSE Cell Lines and Derivative Sublines.** Cell lines designated SVX were morphologically distinct from parental ROSE cells and from sublines containing only the neomycin phosphotransferase gene (neo^R). The capacity of ROSE cell lines and G418-resistant sublines for substrate-independent growth is shown in Fig. 3. Clone 2 and clone 7 SVX sublines form colonies in agarose with relatively high efficiency. The clonogenic capacity of neo^R controls under these conditions was much lower, while parental ROSE cell lines were non-clonogenic. Thus, the SVX sublines demonstrated an enhanced capacity for substrate-independent growth, a classical feature of the transformed phenotype.

Spontaneous transformation is frequently observed after repeated passage of ROSE cells in vitro. It was therefore necessary to determine whether clonal sublines were transformed by mutant RAS or spontaneously transformed. Expression of endogenous and mutant p21^{Hs-RAS} in these cell lines is shown in Fig. 4. Only endogenous p21^{Hs-RAS} expression is seen in neo controls. In contrast, high levels of mutant p21^{Hs-RAS} expression are apparent in clone 7 SVX sublines. Expression of endogenous, but not mutant, p21^{Hs-RAS} was observed in clone 2 SVX sublines, all of which were resistant to G418. The gene for resistance to this compound was contained in the same retroviral vector as the MTRAS fusion gene. Thus, the mutant RAS genes in clone 2SVX sublines were probably not expressed.

These data were consistent with RNA expression (Fig. 5). All SVX sublines demonstrate increased expression of RNA for glucose transporter, compared to neo^R controls. Increased glucose transporter expression is consistent with the transformed phenotype, and with the data in Fig. 3. In contrast, expression of transin, a RAS-associated secreted protease, was observed only in clone 7 SVX sublines. Finally, expression of the MTRAS fusion protein was apparent only in clone 7 SVX sublines, not in clone 2 SVX sublines or neo controls. Together, these data indicate that transformation in clone 7 SVX sublines was associated with expression of mutant RAS, whereas clone 2 SVX sublines apparently spontaneously transformed.
Cisplatin sensitivity of ROSE cell lines and derivative sublines were determined by SRB assay. The cisplatin sensitivity of the RAS-transformed clone 7 SVX 1 and SVX 2 sublines did not differ from parental or neo R controls (Fig. 6A). For comparison, cisplatin sensitivity was also determined in spontaneously transformed clone 2 SVX sublines (Fig. 6B). The clone 2 SVX 2 and SVX 3 sublines were comparable in sensitivity to cisplatin as were parental and neo controls. The clone 2 SVX subline was considerably more sensitive to cisplatin (~10-fold, at 50% inhibitory concentration) than controls.

DISCUSSION

Mutations among the RAS family are among the more frequently observed oncogene abnormalities in human cancer (9, 29). The overall incidence of RAS abnormalities in human tumors has been estimated to be between 10 and 15%. Expression of mutant RAS or overexpression of normal RAS genes can contribute to transformation of some cell lines. Although the cellular mechanisms responsible for RAS effects remain to be fully elucidated, abnormalities within this gene family may have prognostic importance in some human cancers. The frequency with which RAS mutations were detected was increased in high-grade carcinomas of the bladder (12) and prostate (11), and detection of mutant RAS correlated with a worse prognosis among patients with some types of preleukemia (14) or non-small cell lung cancer (13). The relationships between mutant RAS and clinical sensitivity to particular chemotherapeutic agents are undefined.

Resistance to chemotherapy may be related to specific genetic alterations. Mathematical models have been developed which relate clinical resistance to mutations occurring during the process of malignant transformation and progression (6-8). Amplification of specific genes, such as mdr-1, has been detected in cell lines selected for resistance to natural products, respectively (30). In addition, the sensitivity of cell lines to particular cytotoxic drugs can be altered by transfection of specific genes, such as mdr-1 (30).

The specific genes responsible for cisplatin resistance remain to be determined. Some investigators have approached this problem by determining the effects of gene transfection on cisplatin sensitivity. Genes of the RAS family were reasonable candidates given the apparent frequency of RAS abnormalities in human cancer and their association with poor prognosis in some malignancies. Sklar (15) reported that transformation of NIH-3T3 cells by mutational activated ras oncogenes was associated with 4.5-8.5-fold cisplatin resistance. Transformation of the same cell lines by overexpression of normal c-Ha-ras, by v-mos, or by v-fms oncogenes was associated with intermediate levels of cisplatin resistance (~2-fold). Similar data were reported in preliminary form by other investigators (31). Con-
mutant RAS expression in these cell lines were substantial. In the absence of zinc, mutant RAS was nondetectible in the MR4 cell line and its expression was 18-fold higher in the MR7 cell line than in MR5 cells. In addition, induction of mutant RAS expression within individual cell lines did not affect cisplatin sensitivity. Thus, the failure to observe a difference in cisplatin sensitivity among these cell lines is unlikely to reflect selection of cell lines with similar sensitivities from a more heterogeneous population.

Similarly, the cisplatin sensitivity of clone 7 ROSE cell lines transformed by mutant RAS did not differ from controls, despite the substantial levels of mutant RAS expression apparent (Figs. 4 and 5). The effects of induction of mutant RAS expression of a higher level in these cell lines were not determined because both parental and RAS-transformed clone 7 cell lines unexpectedly proved to be very sensitive to cytotoxic effects of zinc. Cisplatin accumulation, glutathione levels, metallothionein levels, DNA platination, and other cellular determinants of cisplatin cytotoxicity were not measured in RAS-transformed sublines because no differences in cytotoxicity were apparent.

It is not surprising that spontaneous transformation of ROSE clone 2 cells was observed. We previously reported that spontaneous transformation of cultured ROSE cells frequently occurs when these cells are repeatedly passaged in vitro (21). Spontaneous transformation under these conditions is consistent with data suggesting that postovulatory surface epithelial proliferation is an important etiological factor in human ovarian carcinogenesis (32). Parental clone 2 and clone 7 cells were isolated and expanded from a mixed population of early-passage, nontransformed ROSE cells. The expansion of these clonal lines required many population doublings. Sublines derived from clone 2 or 7 underwent many additional doublings following G418 selection. The cisplatin sensitivity of spontaneously transformed ROSE cell lines has generally been greater than for corresponding nontransformed parental cells (33).

The greater cisplatin sensitivity observed in some spontaneously transformed ROSE sublines raises an additional issue. It is possible that clone 7 SVX cells were spontaneously transformed and also expressed mutant RAS. Under this circumstance, increased cisplatin sensitivity associated with spontaneous transformation could conceivably be offset by the effects of RAS. We consider this possibility unlikely for several reasons. First, the morphology of clone 7 SVX sublines was consistent with that classically seen in RAS-transformed cell lines, unlike clone 2 SVX sublines. Second, transin expression is a relatively late cellular response to high-level RAS24 expression (19). These conditions should favor RAS transformation. Third, we would not expect the effects of RAS to exactly offset those of spontaneous transformation in different cell lines. Finally, offsetting effects from RAS and spontaneous transformation would be inconsistent with the lack of RAS effect in Rat-1 fibroblast sublines.

Overall, our data failed to disclose an association between expression of mutant RAS and cisplatin resistance in rat fibroblast or ovarian epithelial cell lines. We cannot exclude the possibility that mutant RAS contributes to resistance in isolated cell lines, such as 3T3. This seems unlikely, however, given that RAS genes, and presumably their functions, are highly conserved (34). In any event, the contribution of mutant RAS expression to cisplatin resistance appears to be limited at best.

**ACKNOWLEDGMENTS**

We thank Paul Andrews for suggesting the potential offsetting effects of spontaneous and RAS-induced transformation on cisplatin sensitivity.

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**Fig. 6. Cisplatin cytotoxicity in ROSE clone 7 cell lines (A) and clone 2 cell lines (B), by SRB assay. Points are the means of independent triplicate experiments.**

versely, another laboratory (17) reported that cisplatin sensitivity in clonal NIH-3T3 cell lines transformed by mutationally activated or overexpressed ras oncogenes did not differ from parental controls. One potential criticism of these data, i.e., that differences in cisplatin sensitivity might reflect heterogeneity within the initial cell population, was addressed in the experimental design of Isonishi et al. (16). These investigators compared cisplatin sensitivity of NIH-3T3 cells containing a mutant ras gene linked to a dexamethasone-inducible promoter in the presence or absence of dexamethasone. Thus, cisplatin sensitivity was determined in the same cell population for different levels of ras expression. Dexamethasone produced >10-fold increase in the expression of mutant p21^{Hae-ras}, which was associated with an 8-fold increase in cisplatin resistance. Expression of mutant p21^{Hae-ras} was also associated with a 40% decrease in cisplatin accumulation and a 3-fold increase in metallothionein levels.

The present investigations were an attempt to resolve previous discordant observations and to determine whether mutant RAS genes were more generally associated with cisplatin resistance. Almost all of the previously published work was done in murine NIH-3T3 cells. The relevance of this partially transformed fibroblast cell line was uncertain. In addition, preliminary data from Merriman et al. (18) suggested that RAS-transformed murine mammary epithelial cell lines were actually more sensitive to cisplatin than parental controls. We therefore investigated mutant RAS effects in rat fibroblast and ovarian surface epithelial cell lines.

Expression of mutant RAS did not affect cisplatin sensitivity in MR4, MR5, and MR7 rat fibroblast cell lines. Differences in levels of mutant RAS expression in these cell lines were substantial. In the absence of zinc, mutant RAS was nondetectible in the MR4 cell line and its expression was 18-fold higher in the MR7 cell line than in MR5 cells. In addition, induction of mutant RAS expression within individual cell lines did not affect cisplatin sensitivity. Thus, the failure to observe a difference in cisplatin sensitivity among these cell lines is unlikely to reflect selection of cell lines with similar sensitivities from a more heterogeneous population.
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