Increased Resistance to cis-Diaminedichloroplatinum(II) in NIH 3T3 Cells Transformed by ras Oncogenes

Marshall D. Sklar

Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, Michigan 48109

ABSTRACT

The genetic basis of cellular resistance to the anticancer drug cis-diaminedichloroplatinum(II) (CP) is not well understood. In the course of identifying genes from human tumors capable of conferring resistance to CP, we tested the ability of several types of cellular and viral ras oncogenes (H, K, and N) to alter the CP response of mouse cells. Using clonogenic assays, we found that NIH 3T3 fibroblasts transformed with a missense mutation-activated ras oncogenes demonstrated substantially increased resistance to 1-h exposures to CP (P < 0.05 to < 0.001, at different drug concentrations), with 50% inhibitory concentration ratios (compared to NIH 3T3) of 4.5–8.5. Cells transformed with v-mos v-fms, and with a normal ras protooncogene activated by overproduction driven by an MLV ltr, demonstrate intermediate resistance (50% inhibitory concentration ratio, ~2.0). Cells transfected with the pSV2neo plasmid or with human genomic DNA that is not transforming had survival curves no different from those of NIH 3T3. ras genes are highly conserved in mammalian cells. Should these findings also prove to apply to human tumors, the presence of activated ras genes might help predict clinical response to CP.

INTRODUCTION

The platinum coordination complexes, particularly CP, are drugs of major importance in cancer therapy. As is the case with other drugs, the development of tumor cells resistant to CP is a major problem. At least some of this resistance is thought to have a genetic basis. Several groups have identified specific genes whose activation confers resistance to different drugs. For example, amplification of a specific gene (dihydrofolate reductase) has been implicated in the resistance of tumor cells to methotrexate (1) and the multiple drug resistance family or aberrant expression of a particular gene may not necessarily demonstrate that it is responsible for the differences often observed in drug-induced cytotoxicity among different cell lines. To avoid the inherent difficulties of distinguishing the effects of a single gene in such cells, we decided to test the ability of single transfected oncogenes to alter the CPR of an assay cell line. For our assay cell line, we selected a clone of NIH 3T3 mouse fibroblasts that we had found to be both easily transfected and relatively sensitive to CP. We evaluated CPR by comparing the clonogenic survival of NIH 3T3 cells with NIH 3T3 cells to which single ras oncogenes had been added by transfection. We controlled for nonspecific effects of transfection by testing cell lines transformed by unrelated oncogenes (v-fms and v-mos) and for nonspecific effects of transfection by testing cells transfected with (but not transformed by) the pSV2neo plasmid along with human genomic DNA not containing transforming ras genes. The effects of the transfected genes on CPR were assessed by determining the dose-response survival curves of NIH 3T3 cells and ras-transformed NIH 3T3 cells following exposure to different concentrations of CP for 1 h.

We found that cells transformed by a ras oncogene activated by a missense mutation (c-H-ras, N-ras, v-H-ras, and v-K-ras) were significantly more resistant to CP than untransformed NIH 3T3 cells in the concentration range used. Cells transformed by a normal ras gene transformationally activated by a retroviral ltr and cells transformed by v-fms or v-mos oncogenes had intermediate drug resistance. Untransformed cells transfected with human genomic DNA and the pSV2neo plasmid showed no increase in CP resistance. If these findings should also prove to apply to human tumors, the ability of certain oncogenes to impart resistance to chemotherapeutic agents may be partly responsible for the decreased prognosis of patients whose tumors have certain oncogenic abnormalities.

MATERIALS AND METHODS

Cell Lines. A NIH 3T3 cell line and a Kirsten virus-transformed NIH 3T3 cell line (DT) containing two copies of v-K-ras (7) were obtained from R. Basset. The NIH 3T3 cells used as controls and for all transfections were obtained from D. Lowy, as was NN 192, an NIH 3T3 cell line transformed by transfection with a normal rat c-H-ras protooncogene transformationally activated by linkage with a retroviral ltr [constructed as in DeFeo et al. (8)].

Transfection and Transformation. NIH 3T3 cells were transfected with human tumor genomic DNA or cloned oncogenes (listed in Table 1) by the Wigler et al. (9) modification of the method of Graham and van der Eb (10), as previously described (11). In the case of cells transformed with genomic DNA, only secondary and tertiary transformants were used in this study. Transformed cells were cloned and tested for the ability to grow in soft agar (and, in some cases, in nude mice).

Assay for the Presence and Expression of Transfected Genes. Transformed cells were tested for the presence of the transfected gene by Southern blot analysis (12) using probes for H-ras, N-ras, v-mos, and v-fms. Level of expression was determined by dot-blot analysis using the same probes. DNAs were digested with the EcoRI restriction endonuclease under the conditions recommended by the manufacturer and electrophoresed in 0.8% agarose. Southern blots were performed.
as previously described (11). Probes were labeled by nick translation (13) with biotinylated $d$UTP according to the manufacturer's (BRL) instruction, or with $^{32}P$-labeled dATP as previously described (11). The blots using probes labeled with biotinylated $d$UTP were enzymatically developed using a streptavidin/biotin/calf alkaline phosphatase complex in accordance with the manufacturer's instructions. Blots hybridized with $^{32}P$-labeled probes were autoradiographed as previously described (11). Dot blots were performed on total cellular RNA extracted by the method of Chirgwin (14); 2.5–20 µg RNA from ras-transformed cell spots were spotted on nitrocellulose in a Bio-Rad dot-blot apparatus, baked on, and hybridized with biotinylated pUCEJ6.6 under standard conditions.

Survival Curves/Clonogenic Assay. Cells in exponential growth in Dulbecco's medium with glutamine, pyruvate, antibiotics (penicillin/streptomycin), and 10% fetal bovine serum (D10 medium) were exposed to concentrations of CP varying from 0–40 µM for a period of 1 h. After removing the CP by washing twice with phosphate-buffered saline, the cells were removed with trypsin-EDTA, counted (by hemocytometer or Coulter Counter), and replated in triplicate at several different cell densities (usually, 100–10,000) in 60-mm dishes in D10 medium; 7–10 days after plating, the plates were fixed and stained with 1% methylene blue in 70% methanol, and colonies with 50 or more cells were scored. The surviving fraction of cells plated was plotted against the concentration of CP in the medium. All plots for individual cell lines are the mean of 3–12 separate experimental curves ± 2 SE. The level of statistical significance of differences between the means of experimental points was determined by t test.

RESULTS

Transformation and Characterization of Transformants. All transformed cell lines used in this work formed colonies in soft (0.3–0.35%) agar with plating efficiencies exceeding 10% (12–38). NIH 3T3 and NIH 3T3 transduced but not transformed with other DNAs (human genomic DNA and the pSV2neo plasmid) formed fewer than 1 colony in agar/10⁵ cells plated. Plating efficiencies of transformed and untransformed cell lines in monolayer culture varied from 19–52% (Table 1). Growth rates varied from doubling times of 11–23 h, with no correlation between state of transformation or type of transforming gene and growth rate. Representative cell lines transformed with v-H-ras, c-H-ras, N-ras, and v-fms also formed tumors when injected s.c. or i.p. into nude mice (1–5 million cells), while the parental NIH 3T3 cells did not.

As shown in Fig. 1, the cell lines used in this work that were phenotypically transformed by an oncogene were shown to contain that oncogene by Southern blot analysis. Fig. 1a demonstrates the presence of multiple copies of the c-H-ras oncogene in the 5139 B1-3A cell line (lane 4), the c-H-ras protooncogene in the NN-192 cell line (lane 3), and v-H-ras in the HSV 3A cell line (lane 1). The 23-kilobase endogenous c-H-ras EcoRI fragment is the only fragment seen in DNA from NIH 3T3 (lane 2). Since we have been unable to accurately estimate copy number densitometrically in these enzymatically stained membranes, we used EcoRI digestion to obtain an estimate of copy number, since there is only a single RI restriction site in the pUCEJ6.6 plasmid. In lane 1, the combination of high stringency washes and a c-H-ras probe tend to underestimate the actual copy number for v-H-ras-transformed cells. Since we used calcium phosphate-mediated transfection to prepare these cell lines, the presence of multiple copies was not unexpected. Fig. 1b demonstrates the presence of the HL60 N-ras gene in the HL60 AD2 transformed cell line. Fig. 1, c and d demonstrate the presence of v-fms and v-mos oncogenes in NIH 3T3 cells transformed by these genes. On the dot-blots in Fig. 1, e, the HSV 3A cell line (lane 1) shows substantially elevated production (at least 10-fold) of v-H-ras mRNA compared to NIH 3T3 (lane 1) and two different 5139 (pEJ6.6-transformed NIH 3T3) cell lines (lanes 3 and 4).

Increase of Resistance to CP by Transformation by Missense Mutation-activated ras Genes. We initially compared NIH 3T3 survival curves with those of NIH 3T3 cells transformed by transfection with genomic DNA from the EJ human bladder cancer (c-H-ras) (15) and HL60 promyelocytic leukemia (N-ras) (16) cell lines. To determine whether the effects observed were due to ras oncogenes rather than non-ras genes cotransfected from the human tumors, we also constructed CP dose-response curves for NIH 3T3 cells transformed by transfection with molecularly cloned v-H-ras, c-H-ras, and N-ras genes or transformed by infection with v-K-ras (7). The results are shown in Fig. 2a. The data for the NIH 3T3 cell line were obtained from 12 separate experiments with this cell line and is drawn as the mean ± 2 SE for each point. The curves for the ras-transformed cell lines are the means of multiple experiments for each cell line.

All cell lines transformed by ras genes activated by a missense mutation were significantly more resistant to CP than NIH 3T3 cells at all concentrations of CP tested ($P < 0.01$ to $< 0.0001$ by t test) as shown in Fig. 2. The ratio of that concentration of CP that causes a 50% reduction in survival fraction of a cell line of ras transformants to NIH 3T3 ranged from 4.5–8.5. We observed no significant differences in CPR related to the type of ras gene (H, K, or N, viral or cellular), whether a 12th [EJ (17)] or 61st [HL60 (18)] codon mutation was involved, whether the ras gene was introduced by infection or by transfection, or whether the ras gene was introduced as genomic
CP RESISTANCE: EFFECT OF ras GENES

**Fig. 1.** Presence and expression of transfected DNA in NIH 3T3 cells. For Southern blot, DNA samples (10 µg) from NIH 3T3 cells and NIH 3T3 cells transfected with different oncogenes were digested with EcoRI, electrophoresed in 0.8% agarose, and transferred by Southern blot as previously described (11). The blots were hybridized under high stringency conditions [0.16 standard saline citrate (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4), 60°C final wash] with biotinylated probes (³²P-labeled probe in a) labeled by nick translation and were developed using BRL's BlueGene kit (autoradiography for b). a, pUC3.6.6 (c-H-ras (15)) probe hybridized with DNA from NIH 3T3 cells transformed with various H-ras genes. Lane 1, HSV 3A (NIH 3T3 transformed by v-H-ras, 5 µg DNA); lane 2, NIH 3T3; lane 3, NN 192 (NIH 3T3 transformed by a c-H-ras activated by a retroviral Itr); lane 4, 5139 B1-3A (NIH 3T3 transformed with pUC3.6.6), b, N-ras probe (p52C-1) (16) hybridized with (lane 1) HL60 AD2 (NIH 3T3 cells transformed with HL60 DNA (secondary transformants), (lane 2) NIH 3T3 cells and (lane 3) HL60 DNA; c, v-fms probe (26) hybridized with (lane 1) NIH 3T3 cells and (lane 2) L-fms (NIH 3T3 cells transformed with v-fms), d, v-mos probe (25) hybridized with (lane 1) NIH 3T3 cells and (lane 2) 26407 B1-1 (NIH 3T3 cells transformed with v-mos, pH 10). kB, kilobase(s). For dot blot, e, biotinylated pUC3.6.6 (c-H-ras) probe hybridized with total cytoplasmic RNA (2.5, 5, 10, and 20 µg) from (lane 1) NIH 3T3, (lane 2) HSV 3A, (lane 3) 5139 B1-3A, and (lane 4) 5139 B1-D2 (two different NIH 3T3 cell lines transformed with pUC3.6.6).

DNA or as a gene cloned in a plasmid. The degree of CPR did not depend on the number of ras gene copies in a cell, since cell lines which contains multiple copies of missense mutation-activated ras (Fig. 1a, lanes 1 and 4) have about the same CPR as cell lines containing no more than two copies (e.g., DT[v-K-ras]) (7). The lack of correlation between degree of CPR and H-ras RNA levels on dot blot (Fig. 1e) suggest that the level of expression of the missense mutation-activated oncogene does not have a major effect on CPR.

These results demonstrated the four things: (a) the ability of genomic DNA containing transforming ras genes to transfer resistance shows that specific sequences in the plasmids or viral ltrs were not necessary for conferring CPR; (b) the ability of cloned exogenous genes to transfer resistance indicates that other (non-ras) exogenous human genes that might be present in the secondary and tertiary transformants were not necessary to confer CPR; (c) the similarity of effect of H-, K-, and N-ras genes and of ras genes with 12th and 61st codon mutations suggests that it is the altered ras protein product that is responsible for increased CPR, as the sequences of the different ras genes and mRNAs differ considerably; (d) since the degree of CPR of the transformed cell lines is not greatly affected by the number of gene copies or level of ras oncogene expression, the CPR effect for missense mutation-activated ras genes does not appear to be due to changes in level of expression. Thus, the missense mutations that transformationally activate ras genes also appear responsible for increased CPR.

The effect on CPR of transformation by v-fms, v-mos, and ras protooncogene overproduction is shown in Fig. 2, right. Having observed a striking effect of missense mutation ras genes on CPR, our next step was to determine whether CPR was specifically conferred by mutated ras genes or was a nonspecific effect of transfection or transformation. Since the v-K-ras gene in the DT cell line was introduced by infection, the transfection process itself did not appear necessary to confer CPR. This was confirmed (Fig. 2, right) when no increased CPR was observed in cells transfected with pSV2neo (19) and human genomic DNA not containing a transforming ras gene. (The means and SE for the survival curves of these cell lines do not differ significantly from each other in that of NIH 3T3, so they are not shown separately in the figure). The CPR effects of transformation per se were evaluated by determining the CPR of cell lines transformed with v-mos and v-fms, oncogenes unrelated to ras. Since increased expression of normal ras genes can transform cells (8, 20) and has been associated with advanced stage of cancer (4-6) and hence poor prognosis, we also determined the degree of CPR of an NIH 3T3 cell line transformed with a normal c-H-ras protooncogene activated by overexpression caused by linkage to a retroviral Itr (8, 20).

The single ras curve in Fig. 2, right, represents the mean ± 2 SE for >20 separate survival curves, including the ras curves shown in Fig. 2, left, as well as for other missense mutation ras oncogene-transformed cell lines not shown in the figure. The CPR of cells transformed by missense mutation ras oncogenes differed significantly at all CP concentrations tested (P = 0.05 < 0.0005) from cells transformed by the other oncogenes, including normal c-Ha-ras activated by linkage to a retroviral Itr. Survival of all transformed cells also differed significantly from NIH 3T3 cells at all CP concentrations tested (P < 0.05 to < 0.001). Survival of untransformed cells transfected with non-ras-containing human genomic DNA and pSV2neo plasmids did not differ significantly from that of NIH 3T3 cells (P > 0.3) at the concentrations tested (10-40 µM). Survival curves of cells transformed with v-fms, v-mos, and overproducing c-H-ras did not differ significantly from each other (P > 0.4). Since the means and SEs for the survival curves for these three cell lines were essentially identical, they are combined into a single curve in Fig. 2, right.

**DISCUSSION**

Transformation with missense mutation-activated ras oncogenes significantly increases the CPR of mouse NIH 3T3 cells. As shown in Fig 2, right, cells transformed with genes unrelated to each other (v-fms, v-mos, and an overproducing H-ras protooncogene) also show increased CPR, although to a lesser degree. Thus, while some biochemical process activated or enhanced by transformation per se likely plays a role in the increased CPR of transformed cells, the significantly greater degree of missense mutation ras-mediated CPR argues that a (missense) ras-specific CPR mechanism is also involved. This difference in CPR effect between the two types of ras genes is particularly intriguing and worthy of further investigation. While the greater CPR conferred by missense ras genes may be
The result of greater efficiency of the products of these genes in the same biochemical process, it is also possible that ras oncogenes and protooncogenes may confer CPR by two different pathways, depending on their mechanism of activation.

The biochemical mechanism(s) by which ras oncogenes impart CPR are obscure. The location of the ras protein on the inner surface of the cell membrane (3) suggests that it might function by affecting intracellular drug concentration, possibly by pumping more drug out (CP apparently enters by diffusion; there are no known CP receptors or transport systems) (21) or perhaps by the drug binding to the oncogene protein or to proteins induced by transformation, thus decreasing the bioavailability and distribution of CP to the nucleus and DNA. They might also act by increasing the level of glutathione or other reducing agents/free radical scavengers, which has been associated with increased CPR in some systems (22). Among other possibilities, these oncogenes may selectively affect the expression of genes involved in repair of CP damage (which occurs in the nucleus) (23) or affect genes which alter the structure of chromatin or other nuclear structures to increase access to repair enzymes. Since we have found that mutated ras genes also substantially increase intrinsic resistance to ionizing radiation3 an agent that is certainly not subject to cellular transport mechanisms, we favor a mechanism in which the ras gene protein product specifically but indirectly affects a nuclear process. This argument for a nuclear site of action is of course not decisive, since the mechanisms of resistance to CP and ionizing radiation might prove to be quite different.

While these findings provide evidence that ras oncogenes can increase CPR in one type of cell (NIH 3T3 mouse fibroblasts), the clinical relevance of these findings depends on whether similar effects can be demonstrated in human cells, which is of course speculative at this time. Obviously, the biological differences between NIH 3T3, which is a nonmalignant mouse fibroblast cell line, and the common human epithelial cancers are substantial. Nevertheless, since ras proteins appear to be highly conserved and ubiquitous in eukaryotic cells, we believe it possible that these genes will play a similar role in human cells. This belief has been strengthened by recently obtained preliminary evidence of a similar effect of exogenous ras genes in two human bladder and one human breast cancer cell lines. If so, then these findings suggest certain potentially useful future applications. For example, their presence or aberrant expression in a tumor may help predict whether a patient's tumor will respond to CP. Tumor cells undergo many genetic changes over the course of their evolution from normal cells to highly malignant cells refractory to therapy. Some of these genetic changes likely affect response to therapeutic agents. During the course of carcinogenesis and tumor progression, one characteristic event is activation of oncogenes by several alternate mechanisms (3). If the present findings prove applicable to human tumors, then at least one of the molecular processes of tumor resistance.
progression, oncogene activation, may also impart resistance to therapy by CP and perhaps other cancer therapeutic agents as an inherent part of the natural evolution of the disease. If so, then treatment with CP might be selected for cells with activated ras and other oncogenes. Since activated ras oncogenes have also been associated with an increased metastatic tendency (24), treatment with CP may be selected for tumor cells with greater metastatic tendency as well as greater CP resistance.

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Marshall D. Sklar


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