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Advances in Brief

Modulation of p53 Expression by Human Recombinant Interferon-α2a Correlates with Abrogation of Cisplatin Resistance in a Human Melanoma Cell Line[1]

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Abstract

G3361/CP cells, a cisplatin (CDDP)-resistant subclone of the human melanoma cell line G3361, overexpress wild-type p53 protein and demonstrate an increase in the percentage of cells in G_0–G_1 arrest compared to parental cells. Exposing G3361/CP cells to human recombinant IFN-α2a reduces the high basal levels of p53, releases G3361/CP cells from G_0–G_1 into S phase, and abrogates CDDP resistance. These findings suggest that recombinant IFN-α2a disrupts p53-mediated cell cycle regulation to restore CDDP sensitivity in G3361/CP cells.

Introduction

One possible mechanism by which proliferating neoplastic cells escape drug targeting may be their ability to arrest their growth cycle in response to DNA damage. Cell cycle checkpoints present at G_1-S and G_2 allow the cell to assess DNA integrity before replication (1). Extensive DNA damage may lead to programmed cell death, known as apoptosis (2); however, lesser DNA damage can arrest cells in the growth cycle to allow time for assessment and repair of drug-induced damage (3).

Wild-type p53, also known as the tumor suppressor protein, has been found to mediate cell cycle checkpoints in response to certain types of DNA damage. In particular, agents that produce DNA strand breaks, either directly or associated with replication in the presence of drug-induced DNA lesions, or as a result of excision repair, induce an increase in p53 protein levels (4). Mechanistically, cell cycle control by p53 occurs through its induction of the $p21^{WAF1/CIP1}$ protein (5), which inhibits cyclin E-Cdk2.[3] Once inactivated, cyclin-cdks can no longer phosphorylate target proteins, like the retinoblastoma RB1 protein (6), to release transcription factors, such as E2F1 (7), which are required for G_1 progression into S phase and DNA replication. As a result, cells with increased levels of p53 arrest in G_1 (8). Therefore, p53, through its ability to regulate the cell cycle, may contribute to drug resistance. For this reason, agents that enhance the efficacy of DNA damage by impairing cell cycle checkpoints to disrupt DNA repair are being sought.

It has been known for some time that human rIFN-α2a can enhance the efficacy of CDDP (9), a bifunctional DNA-alkylating agent used against a broad range of malignancies. Indeed, Phase I and II clinical trials using this combination against metastic melanoma, one of the most resistant tumors to chemotherapy (10), demonstrated objective response rates of 15% (11) and 24% (12), respectively. Thus far, however, the mechanism by which rIFN-α2a interacts to enhance CDDP antitumor activity has not yet been defined.

To determine if the enhanced cytotoxicity of rIFN-α2a plus CDDP reflects rIFN-α2a-induced alterations in the DNA damage response pathway, we chose to examine its effects on a CDDP-resistant subclone, G3361/CP, of the human melanoma cell line G3361.

Materials and Methods

Cells and Cell Survival Assays. The G3361/CP subline was developed by repeated acute exposure of its parental cell line to escalated concentrations of CDDP (13, 14); both were a generous gift from Dr. B. A. Teicher (Dana-Farber Cancer Institute, Boston, MA). Resistance of this cloned subline to CDDP correlates with reduced levels of drug accumulation within the cell and an increase in intracellular protein sulfhydryl content and glutathione*S*-transferase activity compared to the parental cell line (13, 14). Additionally, G3361/CP cells demonstrate cross-resistance to other platinum-containing alkylating agents, such as carboplatin and L-tetraplatin, as well as non-platinum-containing antineoplastic agents including mitomycin C and 5-fluorouracil (13, 14). For our studies, subconfluent monolayers of parent or resistant cells, seeded at a concentration of 2×10^4 cells/cm^2, were maintained overnight in RPMI (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.) and antibiotics. Twenty-four hours later, the medium was removed, and the cells were exposed to fresh medium with or without 5000 IU/ml human rIFN-α2a (Roche Laboratories, Nutley, NJ) for 18 h. The following morning, 0–500 μM CDDP (Bristol-Myers, Evansville, IA) was added to the cell cultures for 1 h. After treatment, the cells were washed three times with fresh medium, suspended by treatment with trypsin/EDTA, and reseeded at 3×10^3 cells/cm^2 in duplicate. After 7 days, colonies were stained with crystal violet and counted. Results from three independent experiments are reported as surviving fraction of treated cells normalized to untreated or rIFN-α2a-treated controls. Statistical analysis of results was performed with the Kruskal Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons test using Instat (Graph Pad, San Diego, CA).

Western Blot. Wild-type p53 protein was purified from 1×10^6 parent or resistant cells, after 18 h treatment with or without 5000 IU/ml rIFN-α2a, 5 μM CDDP, or their combination, by microbatch affinity chromatography on the Ab-5 monoclonal p53 antibody (specific for wild-type p53; Oncogene Science, Cambridge, MA) covalently linked to sepharose beads. The human ovarian carcinoma cell line, SK-OV-3 (American Type Culture Collection, Rockville, MD) was used as a negative control. The immune complexes were solubilized by boiling in SDS sample buffer with reducing reagents and resolved by 7.5% SDS-PAGE. Wild-type p53 protein was transferred to a nitrocellulose filter and incubated with the DO-1 p53 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The filter was washed and bound wild-type p53 protein was detected by enhanced chemiluminescence as directed by the manufacturer (Amersham, Arlington Heights, IL). Quantitative comparison of steady-state expression of p53 protein from each treatment group was determined by densitometric analysis (GS 300 Scanning Densitometer, Hoefer Scientific Instruments, San Francisco, CA). As an internal control, equal aliquots from the supernatant of the above immunoprecipitates was resolved by SDS-PAGE and analyzed by Western blotting for actin (Ab-1; Oncogene Science).

Metabolic Labeling and Immunoprecipitation. Subconfluent monolayers of parent and resistant cells were exposed to medium with or without 5000 IU/ml rIFN-α2a for 18 h. The following day, the medium was removed, and cells were methionine depleted in methionine-free RPMI (ICN, Costa Mesa,

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; rIFN-α2a, recombinant IFN-α2a; CDDP, cisplatin.

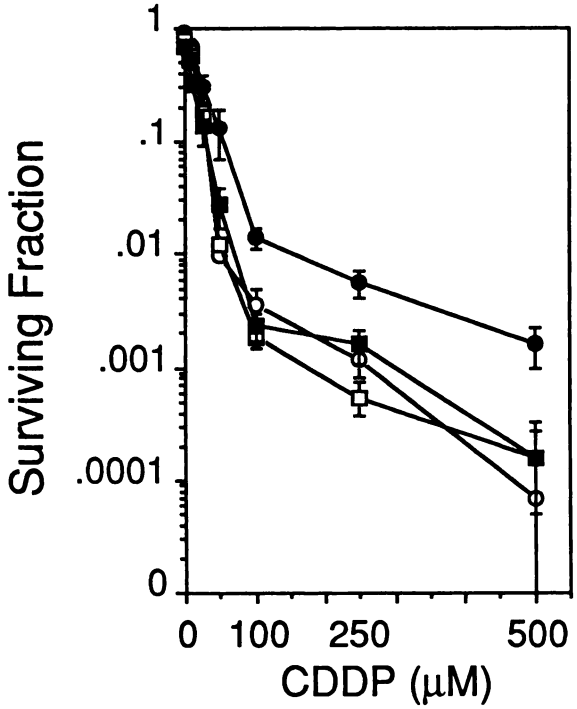

Fig. 1. Survival of the G3361 parent cell line (○ and □) and its CDDP-resistant subclone, G3361/CP (● and ■) after treatment with CDDP (○ and ●) or the combination of 5000 IU/ml rIFN-α2a and CDDP (□ and ■). Subconfluent monolayers of parent or resistant cells were exposed to fresh medium with or without human rIFN-α2a for 18 h. The following morning, 0–500 μM CDDP was added to the cell cultures for 1 h. After treatment, the cells were washed three times with fresh medium, suspended by treatment with trypsin/EDTA, and reseeded in duplicate for colony formation assays. After 7 days, colonies were stained with crystal violet and counted. Results are from three independent experiments and are expressed as the surviving fraction ± SE of cells from treated groups compared to untreated or rIFN-α2a-treated controls.

CA) with or without 5000 IU/ml rIFN-α2a for 30 min before pulsing for 1 h with ^{35}S-methionine (100 μCi/ml; ^{35}S-Trans-Label, ICN) in the presence or absence of 5000 IU/ml rIFN-α2a, 25 μM CDDP, or their combination. Incorporated ^{35}S-methionine was then chased using medium containing 40 mM methionine for 0.75, 4, and 6 h in the absence of drugs. Wild-type p53 protein was immunoprecipitated from equivalent amounts of trichloroacetic acid-precipitable radioactivity on the Ab-5 monoclonal antibody and analyzed by 10% SDS-PAGE as described above. The gel was soaked in Autofluor (National Diagnostics, Manville, NJ) and dried under vacuum, and wild-type p53 protein was visualized by autoradiography using Kodak XAR-5 presensitized film at −70°C overnight.

Flow Cytometry. Parent and resistant cells (1×10^6) were exposed to medium with or without 5000 IU/ml rIFN-α2a for 18 h. The following day, 25 μM CDDP was added to some of the cultures in the presence or absence of 5000 IU/ml rIFN-α2a, and incubation continued for 1 h. After treatment, the cells were washed three times with medium, fresh medium was added, and the cells were incubated for 4 h. Cells from each treatment group were harvested by treatment with trypsin/EDTA, centrifuged, and washed, then resuspended in ice-cold PBS. The cells were stained for 30 min at room temperature in the dark with a solution of 0.1 mg/ml propridium iodide, 0.6% NP40, and 1 mg/ml RNase. After staining, the cell nuclei were disaggregated by passing them through an 18-gauge needle and filtering them through a 30-μm nylon mesh. Flow cytometry was performed by the FACScan (Becton Dickinson, Plymouth, England) using the ModFit LT program (Becton Dickinson). Statistical analysis of results from 3–4 independent experiments was performed with the Kruskal Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons test using Instat.

Results

Survival of each cell line after exposure to CDDP (1–500 μM) for 1 h was determined by colony formation assay (Fig. 1). Exponential survival reflecting inherent sensitivity of G3361 parental cells was maintained through 2.5 logs of killing. Exponential survival of G3361/CP was maintained through 2 logs of killing and represented a 3.5-fold increase in tolerance to drug concentration compared to parental cells ($P < 0.05$). Terminal slopes were apparent in both parent and resistant cells when they were exposed to higher concentrations of CDDP; however, the terminal slope of the G3361/CP cells was more shallow than that of the parent cells, suggesting that a greater number of resistant cells were in a CDDP-insensitive phase of the cell cycle during acute exposure.

Treating resistant cells for 18 h with 5000 IU/ml rIFN-α2a followed by a 1-h coexposure to 1–500 μM CDDP and rIFN-α2a restored exponential survival of G3361/CP cells to 2.5 logs of killing (Fig. 1). Interestingly, this combination treatment reduced the concentration of CDDP required to kill 90% of the resistant cells (IC_{90}) to the same concentration required to produce this effect in parent cells (Table 1). Additionally, combination treatment with rIFN-α2a and CDDP made the survival curve of G3361/CP cells nearly indistinguishable from that of parent cells treated with CDDP or the combination of rIFN-α2a with CDDP ($P > 0.05$).

We next compared wild-type p53 levels in parental G3361 cells and the resistant subline after overnight exposure to rIFN-α2a, CDDP, or their combination by Western blot analysis. Resistant G3361/CP cells expressed approximately 2-fold more intrinsic p53 protein compared to parent cells (Fig. 2). As expected, chronic CDDP treatment (5 μM) induced p53 expression in parental G3361 cells; challenging G3361/CP cells with CDDP (5 μM) produced no increase in p53 expression beyond the basal level. Interestingly, treatment with 5000 IU/ml rIFN-α2a alone inhibited the constitutive expression of p53 in the resistant G3361/CP cells, reducing expression to the level observed in untreated parental G3361 cells. Conversely, rIFN-α2a did not change p53 levels in parent cells in the presence or absence of chronic CDDP and only slightly reduced p53 levels in the resistant cells in the presence of CDDP.

To better understand the relationship of these observations to our cytotoxicity assay, the kinetics of wild-type p53 expression were further examined by ^{35}S pulse-chase analysis after exposure of cells to overnight treatment with rIFN-α2a, 1 h treatment with CDDP, or overnight treatment with rIFN-α2a followed by 1 h coexposure to CDDP (Fig. 3). Detectable levels of metabolically labeled p53 protein were immunoprecipitated from both parent and resistant cells 4 h after

Table 1 *Characteristics of parental G3361 cells and resistant G3361/CP cells in the presence or absence of rIFN-α2a, CDDP, or their combination*

CDDP sensitivity is represented as the concentration required to kill 90 or 99.9% of the cells. Exponentially growing cells were treated with CDDP for 1 h with or without prior overnight exposure (18 h) to rIFN-α2a, then plated for colony formation. For cell cycle analysis, cells were washed after drug treatment, harvested 4 h later, stained with propidium iodide, and analyzed by flow cytometry. Results are the means of 3–4 independent experiments ± SE.

	CDDP sensitivity, μM		Cell cycle phase, % cells	
Cell Line/Treatment	IC_{90}	$IC_{99.9}$	G_0/G_1	S
G3361				
Untreated			54.7 ± 2.7	27.0 ± 4.3
rIFN-α2a			41.9 ± 8.1	43.0 ± 11.0
CDDP	30	50	60.6 ± 3.4	29.4 ± 3.9
rIFN-α2a + CDDP	30	55	57.9 ± 4.4	29.0 ± 6.6
G3361/CP				
Untreated			65.7 ± 2.6[a]	21.3 ± 3.1
rIFN-α2a			47.0 ± 2.7[b]	38.9 ± 4.6[c]
CDDP	60	158	65.3 ± 4.1	24.5 ± 4.5
rIFN-α2a + CDDP	30	80	49.5 ± 3.8[c]	36.4 ± 6.0

[a] $P < 0.03$ compared to untreated parental cells.
[b] $P < 0.003$ compared to untreated resistant cells.
[c] $P < 0.05$ compared to untreated resistant cells.

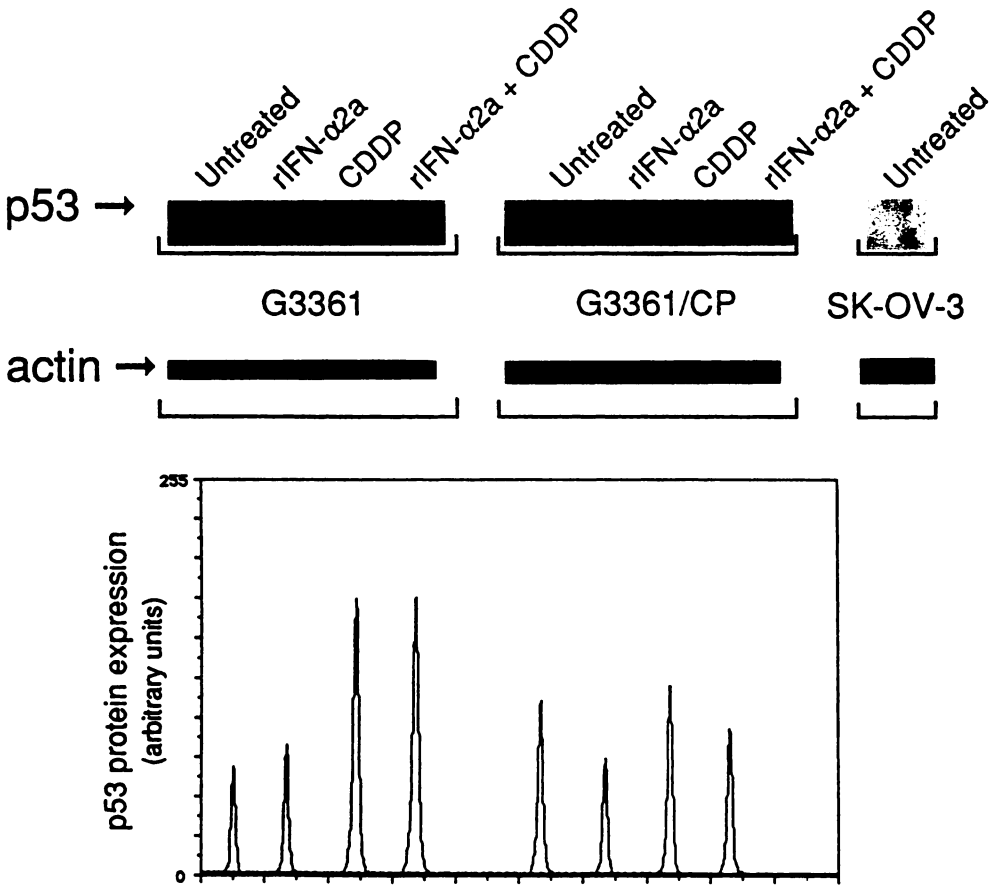

Fig. 2. Western blot analysis of wild-type p53 expression in G3361 parent cells and G3361/CP resistant cells with or without prior exposure to 5000 IU/ml rIFN-α2a (18 h), 5 μM CDDP (18 h), or their combination. Proteins were immunoprecipitated from an equivalent number of cells from each treatment group with antibody specific for wild-type p53 before being analyzed by chemiluminescent anti-p53 immunoblotting. SK-OV-3 cells were included as a negative control. Actin immunoblotting was performed on whole cell lysates from each treatment group to provide an internal control. Results from densitometric analysis of the chemiluminesced p53 proteins are shown.

pulsing. By 6 h, however, there was a 10-fold increase in the amount of p53 immunoprecipitated from resistant cells. This increased amount of ^{35}S-labeled p53 protein suggests that G3361/CP cells possess an enhanced capacity to synthesize p53 protein. Alternatively, these results may reflect a qualitative difference between wild-type p53 protein in parent and resistant cells. Other investigators have reported that posttranslational changes, such as phosphorylation, to wild-type p53 make the protein more recognizable to some monoclonal antibodies during immunoprecipitation (8). Such modifications to the protein may have effects on biological activity, as well as antibody recognition; for example, it has been observed that phosphorylation of p53 by the protein kinase CK2 activates its DNA finding function, which leads to suppression of cellular growth (15).

As expected, a 5-fold increase in ^{35}S-labeled p53 was observed in parent cells treated with CDDP or the combination of rIFN-α2a and CDDP. The observation that under these conditions, rIFN-α2a did not decrease the amount of ^{35}S-labeled p53 protein in parent cells may explain why exposure to CDDP and rIFN-α2a does not increase cytotoxicity in these cells. Interestingly, rIFN-α2a treatment alone increased the amount of ^{35}S-labeled p53 in parent cells, a finding consistent with our previous observations that exposure to rIFN-α2a can produce DNA damage.[4] However, whereas CDDP alone increased expression by 4 h postlabeling, exposure to rIFN-α2a altered the kinetics of p53 expression so that peak levels were not observed until 6 h postlabeling. A similar effect was observed in resistant cells coexposed to rIFN-α2a and CDDP. However, in addition to altering the kinetics of p53 expression, exposing resistant cells to rIFN-α2a decreased by 7-fold the expression of ^{35}S-labeled p53 protein in the presence or absence of CDDP so that ^{35}S-labeled p53 levels were similar to those observed in parent cells treated with the combination. These observations suggest that the difference in endogenous levels of wild-type p53 found in G3361 and G3361/CP cells may contribute, in part, to G3361/CP resistance. Additionally, although treatment with rIFN-α2a appears to inhibit the enhanced expression of endogenous p53 in resistant cells, it does not inhibit the ability of p53 to be induced by exposure to CDDP under these exposure conditions. This later finding suggests that rIFN-α2a may be destabilizing p53 rather than inhibiting its synthesis. Furthermore, this may also explain why rIFN-α2a failed to significantly reduce the expression of wild-type p53 protein in resistant cells chronically exposed to CDDP (Fig. 2).

To assess the functional impact of rIFN-α2a on p53-mediated cellular responses, we next examined rIFN-α2a effects on cell cycle regulation. Cell cycle analysis by flow cytometry demonstrated a significant increase in the percentage of resistant G3361/CP cells in the G_0–G_1 phase compared to parent cells (Table 1), a finding consistent with our observation that the resistant cells overexpress wild-type p53. Also consistent with an increase in p53 levels was the fact that parental cells exposed to CDDP demonstrated a greater percentage of cells in G_0–G_1 arrest. Furthermore, in the G3361/CP-resistant cell line, treatment with rIFN-α2a in the presence or absence of CDDP significantly reduced the percentage of cells in G_0–G_1 and increased the percentage of cells in S phase. Conversely, no significant changes in cell cycle were observed in parent cells treated with rIFN-α2a in the presence or absence of CDDP, although treatment with rIFN-α2a alone did produce a trend toward G_0–G_1 release in parent cells.

[4] J. W. Darnowski, P. A. Davol, and F. A. Goulette. Human recombinant interferon α-2a increases thymidine kinase activity and disrupts DNA repair to synergistically increase 3′-azido-3′-deoxythymidine cytotoxicity in the human colon tumor cell line HCT-8, submitted for publication.

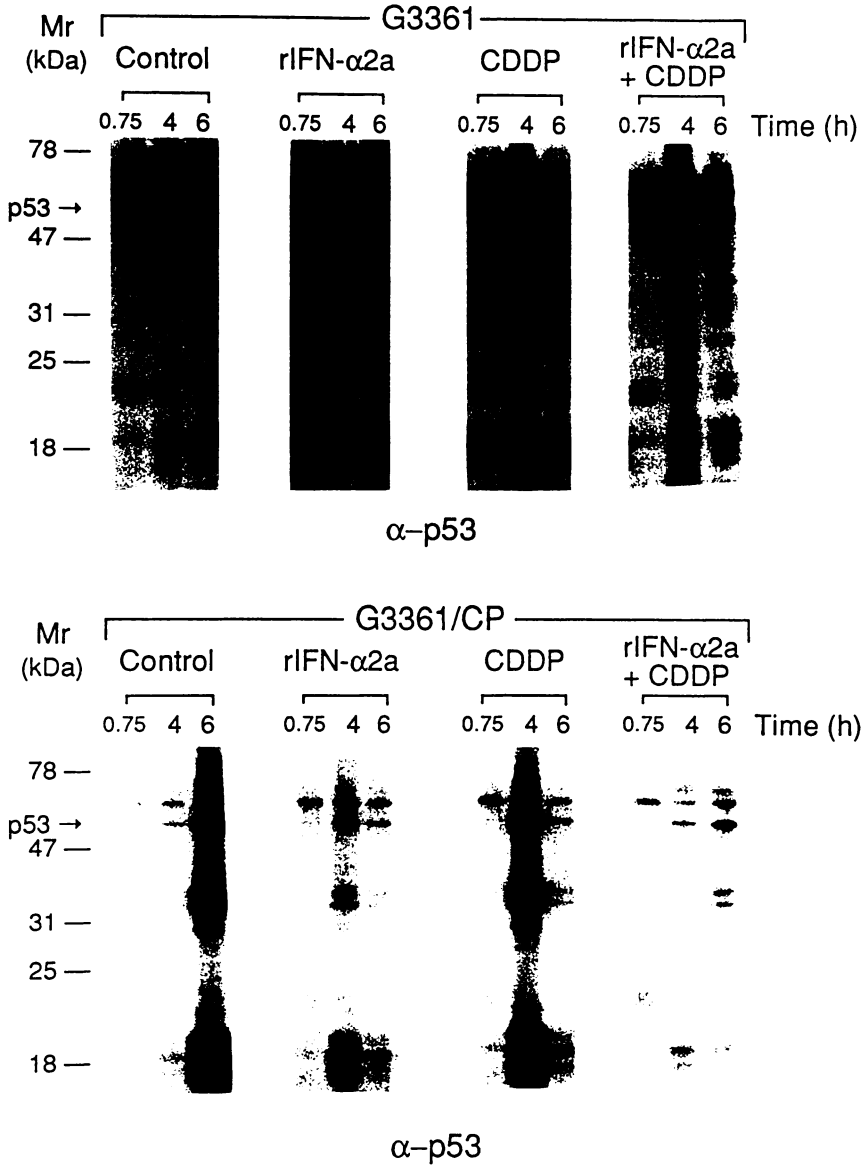

Fig. 3. Time-course expression of pulse-labeled wild-type p53 in G3361 parent cells and G3361/CP resistant cells with or without prior exposure to 5000 IU/ml rIFN-α2a (18 h), 25 μM CDDP (1 h), or their combination. Cells were pulsed with ^{35}S-methionine for 1 h in the absence or presence of drug and then chased with 40 mM unlabeled methionine in the absence of drug for the indicated time period. Proteins, immunoprecipitated from equivalent amounts of trichloroacetic acid-precipitable radioactivity with antibody specific for wild-type p53, were resolved by 10% SDS-PAGE under reducing conditions and were visualized by autoradiography. The positions of the molecular weight standards are indicated. *Arrows*, position of wild-type p53.

Discussion

The discovery that wild-type *p53* acts as a tumor suppressor gene that when malfunctioning, plays a role in the development of neoplasms, has made this gene and its protein product a major area of scientific investigation. Efforts to restore p53 function in cancer cells that harbor mutated or disrupted p53 as a means of stopping the progression of cancer have become the focus of such investigations. Alternatively, an emerging role for p53 is its involvement in cancer chemotherapy and particularly its role in drug resistance. DNA-damaging agents produce cytotoxicity in cancer cells, reflecting their ability to target cells undergoing constant proliferation. It has been observed by some investigators that cell lines exposed to nonlethal doses of DNA-damaging agents increase p53 levels in association with G_1 arrest (16), thus allowing the cells time to assess and repair drug-induced damage. Therefore, p53, through its ability to regulate cell cycle checkpoints, may contribute to treatment failure.

In further support of this concept, the results presented here indicate that overexpression of wild-type p53 in G3361/CP cells is associated with cell cycle arrest and CDDP resistance in this human melanoma cell line. The implications of these results are twofold. First, the significant increase in the percentage of G3361/CP cells in a quiescent phase of the cell cycle may, in part, explain why these cells are less sensitive to CDDP treatment compared to parent cells. These cells may have a slower cycling phase, which protects them from acute exposure to CDDP and allows time for repair of CDDP-induced DNA damage. In fact, G3361/CP cells have an approximately 1.5-fold longer doubling time compared to the parent cells.[5] Interestingly, a similar "protected" state against drug toxicity during chemotherapy has been observed in bone marrow cells treated with granulocyte macrophage colony-stimulating factor, which induces G_0–G_1 arrest in normal hematopoietic stem and progenitor cells (17). Second, the ability of rIFN-α2a to concurrently inhibit p53 expression and release these cells from a quiescent state correlates with an increase in sensitivity to CDDP and, therefore, suggests that its ability to restore CDDP sensitivity to G3361/CP cells may be through targeting this growth regulatory pathway. Interestingly, these same effects on p53 expression and cell cycle kinetics have been observed in myeloid

[5] Unpublished observations.

leukemia and prostatic adenocarcinoma cells treated with caffeine, which also enhances cytotoxicity of many DNA-damaging agents (4).

Currently, the pathway by which cells recognize DNA lesions and respond by inducing p53 to institute cell cycle arrest is not understood. Likewise, the specific mechanism by which rIFN-α2a interacts with wild-type p53 to manipulate cell cycle checkpoints is still under investigation. However, with regard to cancer therapy, the antiproliferative activity of rIFN-α2a has been attributed to its ability to disrupt 2′,5′-oligoriboadenylate synthase, protein kinases, and phosphodiesterases, as well as specific transcriptional and translational factors (18–20). Additionally, we have recently reported that rIFN-α2a can directly inhibit DNA polymerase β,[4], a key enzyme in DNA synthesis and repair. This ability of rIFN-α2a to target various aspects of DNA repair processes to increase DNA damage while concurrently decreasing the expression of p53 may provide clues to the early events that lead to p53 signaling. Additionally, elucidation of the mechanisms of DNA repair processes in relation to p53 cell cycle regulation may provide valuable insight for the purpose of therapeutically manipulating this pathway to improve cancer treatment.

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