Implication of p53 in Growth Arrest and Apoptosis Induced by the Synthetic Retinoid CD437 in Human Lung Cancer Cells

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Abstract

CD437 is a novel retinoid that can induce apoptosis in a variety of tumor cell types by an unknown mechanism. We found that CD437 up-regulated the expression of p21WAF1/CIP1, Bax, and Killer/DR5 and induced G1 arrest and rapid apoptosis in three human non-small cell lung carcinoma cell lines with wild-type p53 but not in five cell lines with mutant p53, suggesting a role for p53 in the effects of CD437. Using HA406 cells in which wild-type p53 protein was degraded by transfection of the human papillomavirus 16 E6 (HPV-16 E6) gene and HA406 cells transfected with a control plasmid only, we found that CD437 increased p53, p21WAF1/CIP1, Bax, and Killer/DR5 in the control transfectants. In contrast, the constitutive p53 protein level was suppressed, and the ability of CD437 to increase p53 and its downstream genes was compromised in E6 transfectants. In addition, CD437 induced G1 arrest and apoptosis in the control transfectants but not in the E6-transfected cells. These results indicate that p53 plays a role in CD437-induced growth inhibition and apoptosis in human non-small cell lung carcinoma cells.

Introduction

p53 is the most frequent target for genetic alteration identified in human cancer, suggesting that the expression of wild-type p53 is often rate-limiting for tumor growth (1). The two important mechanisms by which p53 inhibits tumor cell growth are growth arrest and apoptosis induction (2). It is well documented that the p53 gene product is required for cells to initiate apoptosis in response to genotoxic damage induced by a DNA-damaging agent, including ionizing radiation and certain cancer chemotherapeutic drugs (2). However, the mechanism by which p53 induces apoptosis is thus far largely unknown. It has been proposed that p53 can regulate the expression of several genes that are involved in apoptosis such as Bax (3), p21WAF1/CIP1 (4), and Killer/DR5 (5).

Retinoids are a class of natural and synthetic vitamin A analogues known to play a major role in regulating growth and differentiation of normal, premalignant, and malignant cell types (6). The biological activities of retinoids are thought to be mediated by two classes of nuclear retinoid receptors, retinoic acid receptors and retinoid X receptors, which are members of the steroid hormone receptor gene superfamily (7). Some of the natural and synthetic retinoids show therapeutic and chemopreventive anticancer activity via induction of differentiation and/or apoptosis both in vitro and in vivo (8). Recently, we screened a series of synthetic retinoid receptor-selective retinoids

Materials and Methods

Retinoid. CD437 (17) was synthesized by Galderma (Sophia Antipolis, France). It was dissolved in DMSO at a concentration of 10 mM and stored in the dark at −80°C under N2 atmosphere. Stock solution was diluted to the appropriate final concentrations with growth medium just before use.

Cells and Cell Culture. Human NSCLC cell lines H460, A549, and H1944 that possess wild-type p53 and cell lines H596, H157, H522, Calu-1, and H1792 that have mutant p53 (19) were obtained from Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). These cells were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium supplemented with 5% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air.

Targeting p53 for Destruction. A H460 cell line in which p53 function is lost was generated by transfecting the cells with an HPV-16 E6 expression plasmid. Cells transfected with Neo plasmid were used as a control of cells with wild-type p53 as described previously (20). Individual clones were isolated after selection in the presence of 500 μg/ml G418 (Life Technologies, Gaithersburg, MD). Transfectants were passaged and maintained in the presence of 500 μg/ml G418.

Measurement of Cytochrome c Release. Cells were plated onto 10-cm diameter dishes 1 day before treatment. After the cells were exposed to CD437 for different times, both floating and attached cells were harvested, and cytosolic extracts were prepared as described by Bossy-Wetzel et al. (21) with a slight modification. Briefly, the cells were washed once with PBS and once with buffer A [0.25 M sucrose, 30 mM Tris-HCl (pH 7.9), and 1 mM EDTA]. After a brief centrifugation, the pellets were resuspended in buffer B (buffer A plus the protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) and homogenized with a glass dounce homogenizer and a B pestle (40 strokes). The homogenates were

and found that CD437 was the most active among 37 retinoids tested in inhibiting the growth of retinoic acid-resistant NSCLC cells (9) via induction of apoptosis (10). The apoptosis-inducing activity of CD437 was also observed by us and others in breast cancer (11), melanoma (12), cervical cancer (13), leukemia (14), and lung cancer (10, 15, 16) cells.

Although CD437 can selectively bind to and transactivate the retinoic acid receptor (17), it is thought that this retinoid induces apoptosis through a unique mechanism that is independent of the retinoic acid receptor-mediated pathway, as was demonstrated in lung cancer (10) and breast cancer (11) cells. Several studies have shown that CD437 can increase the level of p53 and G1 arrest; however, it is not clear whether p53 plays any role in CD437-induced apoptosis (11, 15, 16, 18).

In this report, we provide more direct evidence that p53 is involved in CD437-induced apoptosis and G1 arrest in human NSCLC cells by using HPV-16 E6 to target degradation of p53 protein. HPV-16 E6 transfection dramatically diminished not only p53 but also its target genes and CD437-induced G1 arrest and induced apoptosis.

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The abbreviations used are: CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; PARP, poly(ADP-ribose)polymerase; NSCLC, non-small cell lung carcinoma; HPV, human papillomavirus; SRB, sulforhodamine B.

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centrifuged at 14,000 rpm for 30 min, and the supernatants were collected and stored at −80°C. Protein concentration was determined with the Protein Assay kit (Bio-Rad, Hercules, CA). Protein (10 μg) was electrophoresed through a 12% denaturing polyacrylamide slab gel and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. Cytochrome c was detected by Western blotting using mouse monoclonal anti-cytochrome c antibody (7H2,2C12; PharMingen, San Diego, CA) and the enhanced chemiluminescence system (Amersham Life Science, Inc., Arlington Heights, IL) according to the manufacturer’s instructions.

Measurement of CPP32 Activation. Cells were plated onto 10-cm-diameter dishes 1 day before treatment. After the cells were exposed to CD437 for 24 h, both floating and attached cells were harvested and counted. Cells (1 × 10^6) were analyzed for CPP32 (caspase-3) activity using the ApoAlert CPP32 Fluorescent Assay kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions.

DNA Fragmentation Assay. Cells were plated on 10-cm-diameter dishes 1 day before treatment. After a 24-h treatment with CD437, DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) using an ELISA kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. DNA fragments with 3'-hydroxyl ends were determined using an Apo-Direct TUNEL kit (Phoenix Flow Systems, Inc., San Diego, CA) following the manufacturer’s protocol, and DNA ladder formation was analyzed as described previously (22).

Cell Cycle Analysis. Cells were plated on 10-cm-diameter dishes 1 day before treatment. After treatment with 1 μM CD437 for 12 h, the cells were harvested by trypsinization, fixed with cold 70% ethanol, and stored at 4°C. The cells were stained with propidium iodide, and cell cycle was analyzed by flow cytometry as described previously (10).

Growth Inhibition Assay. Cells were seeded at a density of 5000/well in 96-well tissue culture plates 1 day before treatment. After treatment with different concentrations of CD437 for 24 h, cell number was estimated by the SRB assay, and growth inhibition was calculated as described previously (9).

Western Blot Analysis. Whole-cell lysates were prepared as described previously (18), and the protein concentration was determined with the Bio-Rad Protein Assay kit. Protein (50 μg) was electrophoresed through a 10% (for p53 and Bax) or 7.5% (for PARP) denaturing polyacrylamide slab gel and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. Immunoblotting for p53 expression was performed using mouse monoclonal anti-p53 antibody (Ab-6; Calbiochem, La Jolla, CA). PARP cleavage was detected by rabbit polyclonal anti-PARP antibody (VIC 5; Boehringer Mannheim). The blots were developed using the enhanced chemiluminescence system.

Northern Blot Analysis. Total cellular RNA was purified using the Trizol Reagent method (Molecular Research Center, Inc., Cincinnati, OH). Total RNA (20 μg) was electrophoresed in a 0.66 M formaldehyde–1.2% agarose gel and then transferred to a Hybond-N membrane (Amersham) and UV cross-linked. The 1.03-kb EcoRI/Hind III DNA fragment for p21WAF1/CIP1 in GST-CIP1 plasmid obtained from the American Type Culture Collection (Rockville, MD), 0.45-kb EcoRI cDNA fragment for Killer/DR5 in pCR-Killer-Race-6 plasmid described previously (5), 0.6-kb EcoRI cDNA fragment for Bax in pSV-neo vector provided by Dr. S. J. Korsmeyer (University of Washington School of Medical, Saint Louis, MO), and EcoRI/XhoI 340-bp glyceraldehyde-3-phosphate dehydrogenase cDNA (10) were labeled with [32P]dCTP (ICN Pharmaceuticals, Inc., Costa Mesa, CA) to a specific activity of ~2 × 10^9 cpm/μg by the Prime-it II Random Primer Labeling kit (Stratagene, La Jolla, CA). After hybridization for 3–12 h at 68°C in RapidHyb buffer (Amersham), the blots were washed as described previously (9) and then placed against X-ray film (Hyperfilm-MP; Amersham) for autoradiography at −80°C using double intensifying screens. Quantitation was done by Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

Wild-Type p53 Increases Cell Susceptibility to Induction of Apoptosis by CD437 in Human NSCLC Cells. To investigate whether p53 is involved in CD437-induced apoptosis, we first analyzed the effects of CD437 on induction of apoptosis in a group of human NSCLC cell lines with different p53 status. A 24-h treatment with CD437 induced DNA fragmentation (Fig. 1A) in three cell lines (A549, H460, and H1944) with wild-type p53, whereas longer treatment (>48 h) or higher concentrations were needed for CD437 to induce apoptosis in five cell lines (H596, H522, H1792, H157, and Calu-1) with mutant p53 (data not shown). These results indicate that p53 status affects cell response to induction of apoptosis by CD437, i.e., wild-type p53 increases cell susceptibility to induction of apoptosis by CD437 in human NSCLC cells.

Wild-Type p53 Is Associated with G1 Arrest Caused by CD437 in Human NSCLC Cells. A comparison of the effects of CD437 on cell cycle in our set of NSCLC cells lines revealed that all three cell lines with wild-type p53 accumulated in the G1 phase after CD437 treatment, whereas all five cell lines with mutant p53 failed to do so (Table 1). These results indicate that there are defects in G1 arrest in the cell lines with mutant p53, and expression of wild-type p53 is associated with induction of G1 arrest by CD437 in human NSCLC cells.

CD437 Increases the Expression of p53-Regulated Genes p21WAF1/CIP1, Bax, and Killer/DR5 in NSCLC Cells Expressing Wild-Type p53. Similar to the results of apoptosis and G1 arrest induced by CD437, CD437 increased the expression of p21WAF1/CIP1, Bax, and Killer/DR5 genes in three cell lines with wild-type p53 but not in five cell lines with mutant p53 (Fig. 1B), indicating that up-regulation of these genes by CD437 is also dependent on wild-type p53.
Table 1  Cell cycle changes induced by CD437 in human NSCLC cell lines

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<th>Cell line</th>
<th>p53 status</th>
<th>Treatment</th>
<th>G1 (%)</th>
<th>S (%)</th>
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* Mut, mutant; Wt, wild type.

Establishment of a Cell System in Which p53 Protein Level and Its Signaling Pathway Are Disrupted by Transfection of HPV-16 E6. Our previous work (18) and the results described above strongly suggested that CD437-induced apoptosis and G1 arrest could be p53 dependent. To begin to test the hypothesis that wild-type p53 is involved in mediating CD437-induced apoptosis and G1 arrest in some NSCLC cells, we compared and contrasted the expression of p53 in Neo (vector control)- and HPV-16 E6-transfected H460 NSCLC cells grown without or with CD437 by Western blot analysis. Fig. 2A shows that constitutively expressed p53 was present in Neo-transfected cells, and its level was up-regulated by treatment with 1 μM CD437. In contrast, constitutively expressed p53 was decreased and up-regulation of p53 expression by CD437 was completely abolished in HPV-16 E6-transfected cells. These results indicate that p53 levels were indeed decreased in H460 cells expressing HPV-16 E6 and that E6 prevented the increase in p53 in CD437-treated cells.

We next determined whether the targeted loss of p53 protein by E6 affects the expression of some downstream-regulated genes of p53. p21WAF1/CIP1, Bax, and Killer/DR5 mRNAs were up-regulated by CD437 in Neo-transfected H460 (Fig. 2B). However, the up-regulation of these genes by CD437 in E6-transfected cells was almost completely abolished. These results demonstrated that the degradation of p53 protein in E6-transfected cells has led to blockage of CD437-induced expression of the p53-regulated downstream genes.

Disruption of p53 Protein by HPV-16 E6 Expression Abolishes CD437-induced G1 Arrest. Previous studies (15, 16) have shown that CD437 induces G1 arrest in H460 cells and proposed that this was mediated by p21WAF1/CIP1 up-regulation. Because p21WAF1/CIP1 expression induced by CD437 was completely blocked by loss of p53 in HPV-16 E6-transfected H460 cells, we asked whether CD437-induced G1 arrest was affected. Fig. 3A shows that CD437 induced G1 arrest only in Neo-transfected H460 cells but not in HPV-16 E6-transfected cells, indicating that E6 expression has also led to loss of CD437-induced G1 arrest.

Disruption of p53 Protein by HPV-16 E6 Expression Blocks CD437-induced DNA Fragmentation. We hypothesized that if p53 plays a role in mediating CD437-induced apoptosis, destruction of p53 function by E6 should affect cell sensitivity to induction of apoptosis by CD437. Therefore, we examined the responsiveness of E6-transfected H460 cells to CD437 treatment in comparison with that of Neo-transfected cells. Using different approaches, we found that CD437-induced DNA fragmentation was decreased in E6-transfected H460 cells (Fig. 3B). This was evidenced by a decrease in DNA ladder formation using agarose gel electrophoresis, by a decrease in cytoplasmic histone-associated DNA fragments using ELISA, and by a decrease in DNA breaks with 3' hydroxyl ends using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-flow cytometry analysis. Furthermore, the two cell variants exhibited a considerable difference in response to growth inhibition and apoptosis induction by CD437 (Fig. 3C). These results clearly indicate that degradation of p53 protein by E6 expression blocks CD437-induced apoptosis in H460 cells.

Disruption of p53 Protein by HPV-16 E6 Expression Suppresses CD437-induced CPP32 Activation and PARP Cleavage. An activation of caspases, especially that of CPP32 (caspase-3), is another hallmark of apoptosis (23). CPP32 activity was increased 10-fold (Fig. 4A), and CPP32-cleaved M, 89,000 fragment of the substrate PARP was observed (Fig. 4B) in Neo-transfected but not in E6-transfected H460 cells, demonstrating that E6 expression suppresses CD437-induced, apoptosis-related protease activity in H460 cells.

Disruption of p53 Protein by HPV-16 E6 Expression Suppresses Release of Cytochrome c into the Cytoplasm by CD437. Recently, release of mitochondrial cytochrome c has been linked to the activation of CPP32 and the triggering of apoptotic DNA fragmentation (24, 25). Because CD437 strongly activates CPP32 and induces DNA fragmentation in H460 cells, we further compared the effects of CD437 on the release of mitochondrial cytochrome c in Neo-transfected and E6-transfected H460 cells. As shown in Fig. 4C, CD437 increased cytosolic cytochrome c as early as 6 h in Neo-transfected but not in E6-transfected cells, indicating that E6 expression interrupts early signaling for CD437-induced DNA fragmentation.

![Fig. 2. Effects of HPV-16 E6 expression on the level of p53 and p53-regulated genes p21WAF1/CIP1, Bax, and Killer/DR5 in H460 cells grown in the presence or absence of CD437. After 15-h treatment with 1 μM CD437, cells were harvested for preparation of whole-cell protein lysates for Western blot analysis (A) and total RNA for Northern blot analysis (B) as described in “Materials and Methods.” GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](Image 351x105 to 517x376)
In this study, we compared the effects of CD437 on apoptosis induction and G1 arrest in eight human NSCLC cell lines with different p53 status. CD437 treatment led to G1 arrest and rapid apoptosis in three cell lines with wild-type p53 (H460, A549, and H1944) but not in five cell lines with mutant p53 (H596, H522, H157, H1792, and Calu-1). Moreover, the increase in the expression of downstream target genes of p53 such as p21WAF1/CIP1, Bax, and Killer/DR5 by CD437 also occurred only in these three cell lines with wild-type p53. These results strongly support the hypothesis that the p53-signaling pathway is involved in mediating CD437-induced apoptosis in some NSCLC cells.

The HPV-16 E6 gene product has been shown to facilitate degradation of p53 in a ubiquitin-dependent pathway (26), rendering the cells effectively p53-minus. Human NSCLC H460 cells have wild-type p53 (19). Here we confirmed that the level of this p53 is diminished after these cells were transfected with E6. We found previously that p53 protein can be up-regulated by CD437 treatment of H460 cells (18). In this study, we demonstrated that CD437 failed to induce p53 expression in H460 cells transfected with HPV-16 E6. This provided us with a good system to investigate the role of p53 in CD437-induced apoptosis in human NSCLC cells. Using this system, we obtained several lines of direct evidence indicating that p53 is indeed involved in mediating CD437-induced apoptosis in H460 cells: (a) CD437-induced DNA fragmentation was blocked in HPV-16 E6-transfected cells; (b) CD437 failed to induce CPP32 activation and subsequent PARP cleavage in cells transfected with HPV-16 E6; and (c) CD437 failed to induce the release of cytochrome c from mitochondria in HPV-16 E6-transfected H460 cells. It is believed that the biochemical hallmark of apoptosis is the cleavage of chromatin into nucleosomal fragments. Recently, the findings that cytochrome c release and caspase activation are involved in the induction of DNA fragmentation (24, 25) have increased our understanding of apoptosis. The release of cytochrome c from mitochondria appears to trigger the activation of CPP32 by binding to Apaf-1 (27). The activated CPP32...
then cleaves and activates the $M_{65}$, 45,000 subunit of DFF, which in turn leads to the degradation of DNA into nucleosomal fragments (25).

p53 has been implicated in a variety of cellular processes (2). However, the most extensively studied and, perhaps, undisputed roles of p53 are to induce growth arrest and to induce apoptosis (2). These effects are thought to be mediated by genes that are regulated by p53. The growth arrest is at least in part mediated by transcriptional activation of $p21$WAF1/CIP1, which binds to and inactivates the cyclin-dependent kinases required for cell cycle progression (2). G1 arrest induced by CD437 in human cancer cell lines was observed previously by others (11, 15, 16). In the present study, we confirmed that CD437 was able to cause G1 arrest in several NSCLC cell lines with wild-type p53, which correlated with the up-regulation of p21 expression by CD437 in these cell lines. Furthermore, we found that targeted degradation of p53 protein by HPV-16 E6 led to the loss of CD437-induced G1 arrest in H460 cells, perhaps by abolishing the up-regulation of $p21^{WAF1/CIP1}$ expression by CD437. In addition, we found that cell lines with mutants tend to accumulate in S phase after exposure to CD437 (Table 1). This could be related to the loss of normal function of p53 and the inability of CD437 to up-regulate $p21^{WAF1/CIP1}$ expression in these cell lines. Therefore, we conclude that CD437-induced G1 arrest in NSCLC cells was mediated by p53 through the activation of $p21^{WAF1/CIP1}$.

It has been suggested that p53 transcriptional activity may be necessary for apoptosis in some systems (28, 29). Bax is the first proapoptotic protein shown to be a direct transregulational target of p53 (3). Unfortunately, no clear evidence exists for a straightforward link between p53-mediated Bax expression and apoptosis. Several recent studies have shown that p53-induced apoptosis occurs in the absence of increases in Bax mRNA and/or protein levels (2, 29), and cells from bax-deficient mice show a normal p53-dependent apoptotic response after exposure to ionizing radiation (30), suggesting that Bax is not absolutely required for p53-induced apoptosis. Recently, the Killer/DR5 gene was identified as a novel p53-modulated death receptor gene (5), which may play an important role in p53-mediated apoptosis.

We observed that the expression of both Bax and Killer/DR5 genes was up-regulated in H460 cells transfected with the Neo gene, as we found in three NSCLC cell lines with wild-type p53 but not in the cells transfected with HPV-16 E6 gene or NSCLC cell lines with mutant p53, indicating that CD437 up-regulates the expression of both genes through p53. Our results also support the observation by Wu et al. (5) that Killer/DR5 is a p53-regulated gene. Therefore, we suggest that Killer/DR5 and possibly Bax genes may mediate CD437-induced, p53-dependent apoptosis in human NSCLC cells.

In human breast cancer cells, Shao et al. (11) reported that CD437 induced G0-G1 arrest and apoptosis via regulation of $p21^{WAF1/CIP1}$, Bcl-2, and Bax in a p53-independent manner. Adachi et al. (15) and Li et al. (16) reported that CD437-induced apoptosis in some lung cancer cells did not necessarily require p53. It appears, therefore, that in some cells CD437 can induce apoptosis in a p53-independent pathway, either by inducing downstream genes of p53 (e.g., $p21^{WAF1/CIP1}$ or Bax) or by an as yet unknown mechanism. In contrast, our results using cells in which p53 was targeted for destruction strongly suggest that the rapid CD437-induced apoptosis in NSCLC cells involves a p53-dependent mechanism. Interestingly, even HPV-16 E6-transfected H460 cells eventually succumb to CD437-induced apoptosis after a prolonged treatment (data not shown), suggesting that p53 may be required for a rapid execution of the apoptosis program, whereas a slower apoptosis may occur via a p53-independent pathway.

References

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