Restoration of p53 Functions Protects Cells from Concanavalin A–Induced Apoptosis

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Abstract

A great majority of human cancers encounter disruption of the p53 network. Identification and characterization of molecular components important in both p53-dependent and p53-independent apoptosis might be useful in developing novel therapies. Previously, we reported that concanavalin A (Con A) induced p73-dependent apoptosis of cells lacking functional p53. In the present study, we investigated the mechanism and role of p53 in protection from apoptosis induced by Con A. Treatment with Con A resulted in apoptosis of p53-null ovarian cancer, SKOV3, or Li-Fraumeni syndrome, MDAH041 (041), cells. However, their isogenic pairs, SKP3 and TR9-7, expressing wild-type p53 were much less sensitive and were protected by G1 arrest. Inhibition of p53 function rendered these cells sensitive to Con A. Con A–induced apoptosis was accompanied by upregulation of forkhead box O1a (FOXO1a) and Bcl-2–interacting mediator (Bim), which were strongly inhibited after p53 expression and rescued after p53 ablation. Moreover, ablation of Bim by short hairpin RNA protected cells from apoptosis. Taken together, our study suggests that Con A induces apoptosis of cells lacking p53 by activating FOXO1a-Bim signaling and that expression of p53 protects these cells by inducing G1 arrest and by downregulating the expression of both FOXO1a and Bim, identifying a novel cross-talk between FOXO1a and p53 transcription factors.

Introduction

In normal cells, the transcription factor and tumor suppressor protein p53 plays a pivotal role in controlling the cell cycle, apoptosis, genomic integrity, and DNA repair in response to various forms of genotoxic stress. The p53 protein is regulated by complex posttranslational modifications, such as phosphorylation and acetylation, which contribute to its stabilization and activation (1–5). It is widely accepted that differential modification of p53 differentially regulates patterns of gene expression, which then drive distinct biological responses. DNA damage (ataxia-telangiectasia mutated and checkpoint kinase 2), aberrat growth signals (p14ARF), and chemotherapeutic drugs can activate and stabilize p53 (6–9). Four functional groups of genes are transcribed by p53, including cell cycle inhibition (p21, reprimo, growth arrest and DNA damage 45, and 14-3-3), apoptosis (p53 apoptosis effector related to PMP-22, NOXA, p53 upregulated modulator of apoptosis (PUMA), p53-regulated apoptosis-inducing protein 1, apoptosis-stimulating protein of p53 1/2, Fas, Bax, and p53-induced protein with death domain), genomic stability (p21, DNA damage-binding protein 2, MIF5 protein homologue 2, and xeroderma pigmentosum group C), and inhibition of angiogenesis (thrombospondin-1, maspin, brain-specific angiogenesis inhibitor 1, and glioma-derived angiogenesis inhibitory factor; refs. 6–10). Accumulating evidence suggests that p53 can also act as a transrepressor. Transcriptional repression of several genes by p53 is critically important for carrying out its functions (11–13). Due to these inevitable roles, p53 has been considered as the “molecular guardian” of the genome. Unfortunately, p53 is mutated in ~50% of human cancers and functionally inactivated in a further 20% (14–17). Moreover, toxicity of chemotherapy is always an important issue because most currently available chemotherapeutic drugs kill not only the cancer cells but also the normal cells. From the therapeutic point of view, it is therefore important to devise strategies whereby cancer cells defective in functional p53 are eliminated by p53-independent apoptosis and, simultaneously, normal cells with functional p53 are protected by reversible growth arrest in a p53-dependent manner.

In mammals, the forkhead box O (FOXO) transcription factors consist of four members—FOXO1, FOXO3, FOXO4, and FOXO6—and are emerging as key regulators of many cellular functions, including metabolism, cell proliferation, cell survival, and life span (18, 19). Like p53, the activity of FOXOs is tightly controlled by posttranslational modifications, including phosphorylation (Akt and non-Akt mediated), acetylation, and
ubiquitylation. Similar to p53, activation of FOXOs leads to cell cycle arrest or apoptosis, depending on the cellular context and type of stress, and both these transcription factors share several common downstream target genes, such as p21, p27, death receptor, FasL, and PUMA (18–21). Whereas accumulating evidence suggests that p53 and FOXO may share some parallel functions, some recent reports show cross-talk between these two transcription factors. The activation of p53 led to FOXO3a phosphorylation, cytoplasmic localization, and inhibition of transcription activity in response to DNA damage (22). In contrast, activation of FOXO3a by serum starvation or by expressing a constitutively active FOXO3a stabilized p53, inhibited p53 transactivation, and could induce p53-dependent apoptosis in a transcription-independent manner (23).

Concanavalin A (Con A) is a plant lectin obtained from jack beans, which cross-links cell surface glycoproteins, thereby initiating various cellular responses, including T-cell activation and apoptosis (24–26). Con A also exhibits cell agglutination and mitogenic activities (25, 27). Previously, we have reported that Con A induced p73-dependent apoptosis of cells lacking functional p53 by transcriptionally upregulating the expression of both FOXO1a and its proapoptotic target Bcl-2–interacting mediator (Bim) of cell death (28). In the present study, we investigated the role and mechanism of p53 in protection of cells from Con A–induced apoptosis. We find that p53-dependent arrest precludes the p73-dependent apoptosis in response to Con A. Moreover, we provide evidence for a novel cross-talk between p53 and FOXO1a, in which p53 transcriptionally represses the expression of FOXO1a and Bim and thus protects cells from Bim-mediated apoptosis.

Materials and Methods

Cell Culture and Cell Treatment
All cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO2 incubator. For Con A treatment, either 3 × 10^5 (041 and TR9-7) or 4 × 10^5 (SKOV3 and SKP53) cells were plated in a 10-cm culture dish. After overnight incubation, the medium was replaced with fresh medium and the cells were treated with either 15 μg/mL (041 and TR9-7) or 20 μg/mL (SKOV3 and SKP53) of Con A for the indicated times.

Materials and Chemicals
Con A was purchased from Calbiochem. Anti-p53 (DO-1) and anti-p21 antibodies were purchased from Santa Cruz Biotechnology, anti-FOXO1a was from Bethyl Laboratories, and anti-Bim was from Abcam. Antibodies that recognize cleaved poly(ADP-ribose) polymerase (PARP) and phospho-p53 were purchased from Cell Signaling Technology, and the p53 inhibitor pifithrin-α (PFT) was from Sigma-Aldrich.

Generation of Viruses and Infection of Mammalian Cells
Lentiviral short hairpin RNA (shRNA) constructs, pLVGFP and pLVp53, were kind gifts from Dr. Didier Trono (National Center of Competence in Research, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). These constructs were used to generate virus per standard protocols from Dr. Trono. In brief, 293T cells were transfected with 1 μg pMD2G, 1.6 μg pCMV-ΔR8.74, and 3 μg of the lentiviral construct using Lipofectamine Plus reagent. Viruses thus produced were used to infect TR9-7 cells overnight, after which the cells were allowed to recover in virus-free culture medium for an additional 72 h and then used for experiments. Construction of plasmids for shBim and scrambled shRNA (control) was described elsewhere (29). Phoenix cells were transfected with the constructs to generate retroviruses, which were then used to infect mammalian cells. Clones were selected by puromycin selection.

Cell Cycle Analyses
The DNA content of trypsinized cells was determined by staining with propidium iodide. Cells were fixed overnight in 90% methanol. The fixed cells were washed twice with cold PBS and stained with propidium iodide containing RNase. Cell cycle distribution was determined by using a flow cytometer.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay
For the apoptosis assay, cells were grown in 10-cm culture dishes and treated with Con A for the indicated times. The cells were fixed in 70% ethanol and labeled with fluorescein-tagged bromo-dUTP and propidium iodide per the manufacturer’s protocol using the APO-bromodeoxyuridine (BrdUrd) apoptosis kit (Phoenix Flow Systems).

RNA Isolation and Real-time PCR
Total RNA was extracted from the cells by using a Qiagen RNeasy Mini kit according to the manufacturer’s protocol. Amplification of the corresponding gene was done using primer sets supplied by Applied Biosystems, Inc. The data were analyzed for fold induction of each gene compared with the untreated sample after normalization with β-actin expression.

Western Blot Analyses
Total cellular proteins were isolated by lysing the cells in 20 mmol/L Tris-HCl (pH 7.5), 2% (w/v) SDS, 2 mmol/L benzamidine, and 0.2 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were determined by the Bradford method (Bio-Rad). Proteins were resolved on SDS–10% polyacrylamide gels and then transferred to polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat skimmed milk and incubated

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with the respective antibody followed by incubation with a secondary antibody. Proteins were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) as directed by the manufacturer.

Results

p53-Mediated Cell Cycle Arrest Protects Cells from Con A-Induced Apoptosis

The tumor suppressor protein p53 plays a critical role in cell cycle arrest and apoptosis induced by most chemotherapeutic agents. Unfortunately, p53 is mutated in 50% of all human cancers and functionally inactivated in a further 20% (14–17). In search for an agent that can selectively induce apoptosis of cells lacking functional p53 while protecting those expressing wild-type p53, we developed two isogenic cell pairs that differ only in their p53 status. The p53-null 041 cells were originally obtained from a postcresis patient with Li-Fraumeni syndrome. Expression of wild-type p53 was restored by a tetracycline-regulated promoter, and the derived cells, TR9-7, express a low level of p53 in the presence of tetracycline, withdrawal of which induces substantial expression of p53 and many of its target proteins, including p21, and induces both cell cycle arrest (30) and apoptosis (31). We also introduced wild-type p53 into the p53-null ovarian cancer cell line SKOV3. Recently, we reported that Con A treatment leads to p73-mediated apoptosis in cells lacking p53 by regulating FOXO1a and Bim expression (28).

To investigate the role of p53 in Con A–induced apoptosis, both the cell pairs were treated with Con A for 4 days. Photographs of the cells were captured with a phase-contrast microscope (Fig. 1A). After Con A treatment, a great majority of 041 and SKOV3 cells adopted a rounded shape and detached from the surface, whereas TR9-7 and SKP53 cells remained attached to the surface with normal morphology. To quantify and determine the mode of cell death, we did terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays after 4 days of Con A treatment. As shown in Fig. 1B, Con A treatment induced apoptosis of cells lacking functional p53 (041 and SKOV3). In contrast, restoration of p53 functions in these cells protected them from Con A–induced apoptosis. Degradation of PARP by Con A treatment in SKOV3 cells and its inhibition by p53 also suggest p53-mediated protection from Con A–induced apoptosis (Fig. 1C).

We next investigated the cell cycle profile of 041 and TR9-7 cells after Con A treatment for different time periods as described in Materials and Methods. Con A treatment induced an initial G2-M delay followed by G1 arrest in both 041 and TR9-7 cells (Table 1). Although 041 cells subsequently underwent apoptosis, TR9-7 cells gradually accumulated in G1. After 72 hours of treatment with Con A, >91% of SKP53, but not SKOV3, cells were also arrested in G1 (Fig. 1D). These results suggest that Con A induced apoptosis of cells lacking p53, whereas cells with p53 were protected by arresting in G1 phase of the cell cycle.

Figure 1. p53 protects cells from Con A–induced apoptosis. A, both the cell pairs were treated with Con A (15 μg/mL for 041 and TR9-7; 20 μg/mL for SKOV3 and SKP53). Photographs of phase-contrast microscopy images were captured after 96 h of Con A treatment. B, after 96 h of treatment with Con A, cells were fixed in 70% ethanol and stained with APO-BrdUrd for apoptotic cells and sorted by flow cytometry. C, SKOV3 and SKP53 cells were treated with Con A for the indicated times. Total cell lysates were immunoblotted with anti-PARP, which detects only the degraded PARP at 85 kDa. D, after 72 h of treatment with Con A, SKOV3 and SKP53 cells were fixed in 90% methanol, stained with propidium iodide, and sorted by flow cytometry for cell cycle distribution.
Con A Modulates Phosphorylation of p53 without Changing p53 Protein Level

As cells expressing p53 were rendered resistant to Con A–induced apoptosis, we next investigated the activation of p53 by Con A. Cells were treated with Con A and total cell lysates were immunoblotted with anti-p53. As shown in Fig. 2A and B, Con A treatment did not increase the expression of p53 protein in either cell line. For transcription factors, posttranslational modification is very important and sometimes sufficient to increase their transcriptional activity. We next examined the phosphorylation of p53 at the major phosphorylation sites using antibody against phospho-p53 (Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392). Treatment with Con A increased the phosphorylation of p53 at Ser9, Ser15, Ser20, and Ser46 (Fig. 2C). The kinetics of these phosphorylation events was corroborated with the induction of p53 target genes p21 and p27. No phosphorylation was observed at Ser37 and Ser392. These results indicate that Con A activates p53 by modulating its phosphorylation status without changing the protein level.

p53 Is Critical for Protection against Con A

We next investigated whether the protective effect against Con A is mediated via p53 using PFT, a known p53 inhibitor. TR9-7 cells were pretreated with 15 μmol/L PFT for 3 hours followed by treatment with Con A for 72 hours in the presence of PFT. To examine any effect of PFT on cellular growth, cells treated with PFT without Con A were used as control. Methylene blue staining of the cells revealed that PFT alone had no remarkable effect on cellular growth but further inhibited the growth

Table 1. Cell cycle distribution of 041, TR9-7, and TR-shp53 cells after Con A treatment

<table>
<thead>
<tr>
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<td>Sub-G1</td>
<td>G1</td>
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NOTE: Cell cycle profiles of 041, TR9-7, and TR-shp53 cells. Cells were treated with 15 μg/mL Con A for the indicated times. After methanol fixation, cells were stained with propidium iodide and analyzed by flow cytometry.

Figure 2. Con A modulates phosphorylation status of p53 without changing p53 protein level. A and B, both the cell pairs were treated with Con A for the indicated times. Total cell lysates were treated with anti-p53 antibody. C, SKP53 cells were treated with Con A for the indicated times. Total cell lysates were subjected to Western blotting with anti–phospho-p53 (Ser9, Ser15, Ser20, Ser37, Ser392, and Ser392), anti-p53, anti-p21, and anti-p27.
of cells in the presence of Con A (Fig. 3A). Photographs of cells captured 72 hours following Con A treatment showed that inhibition of p53 by PFT rendered the cells sensitive to Con A (Fig. 3B). TUNEL staining also revealed that PFT alone could not induce any apoptosis. However, PFT pretreatment of these cells, which were otherwise resistant to apoptosis, drastically induced apoptosis following Con A treatment (Fig. 3C). Finally, we checked the effect of PFT on the expression of p53 and its target p21 and on degradation of PARP. As shown in Fig. 3D, PFT pretreatment did not have any effect on the expression of p53 but strongly inhibited Con A–induced expression of p21 in cells with p53. PFT pretreatment failed to inhibit Con A–induced p21 induction in 041 cells, which was induced in a p73-dependent manner (28). PFT pretreatment also induced PARP degradation.

Figure 3. p53 is required for the protective effect against Con A. A, TR9-7 cells were treated with 15 μmol/L PFT for 3 h followed by treatment with Con A for 72 h in the presence of PFT. Plates were stained with methylene blue. The intensity of staining was quantified by dissolving the dye in 0.1N HCl. Columns, average of three independent experiments; bars, SD. B, TR9-7 cells were treated as in A and photographs were captured by phase-contrast microscopy. C, TR9-7 and SKPS3 cells were pretreated with PFT followed by treatment with Con A for 72 h and APO-BrdUrd staining for TUNEL-positive cells. D, cells were pretreated with PFT for 3 h followed by treatment with Con A for 48 h (TR9-7) and 72 h (SKPS3 and 041). Total cell lysates were immunoblotted with antibodies against p53, p21, and cleaved PARP.
Together, these results suggest that p53 protects cells from Con A–induced apoptosis.

To further confirm the protective role of p53 against Con A–induced apoptosis, we ablated the expression of p53 in TR9-7 cells by transducing shp53. Cells transduced with shGFP (control) or shp53 were treated with Con A, and expression of p53 and p21 was measured by Western blotting. The transduction of shp53 nicely ablated the expression of p53 and the basal level of p21 (Fig. 4A). p21 expression after Con A treatment was also reduced but not completely. The residual p21 expression after p53 ablation might result from p53-independent sources, as in 041 cells (28). Photographs of these cells were also taken 72 hours after Con A treatment. As shown in Fig. 4B, cells transduced with shGFP remained alive with some degree of growth inhibition, whereas those with shp53 started dying and became rounded. Substantial increase in the sub-G1 population also suggests that ablation of p53 renders the cells sensitive to Con A (Table 1).

**p53-Mediated Inhibition of FOXO1a-Bim Signaling**

We have previously reported that Con A induced p73-dependent expression of FOXO1a and Bim in cells lacking p53 (28). We next examined the effects of p53 on the expression of FOXO1a and Bim in response to Con A. Both the cell pairs were treated with Con A, and expression of FOXO1a and Bim was analyzed by Western blotting and real-time PCR. As shown in Fig. 5A to C, Con A treatment efficiently induced FOXO1a and Bim at both the mRNA and protein levels in cells lacking p53. However, rescue of p53 function in the same cells dramatically inhibited the expression of FOXO1a and Bim. To unambiguously confirm that the inhibition of FOXO1a and Bim expression was due to p53, we next examined the expression of FOXO1a and Bim in TR9-7 cells transduced with shGFP and shp53. There was little or no increase in the levels of FOXO1a and Bim proteins in response to Con A in cells transduced with shGFP (Fig. 5D). However, ablation of p53 markedly increased the expression of both FOXO1a and Bim. These results again suggest that p53 inhibits FOXO1a signaling in response to Con A. Finally, we studied the role of Bim expression in Con A–induced apoptosis. Expression of Bim was ablated in 041 cells by transducing shBim from a retroviral-based expression vector. Clones in which the expression of Bim was ablated were isolated after drug selection (Fig. 6A). Photographs taken after 72 hours of Con A treatment (Fig. 6B) or TUNEL assay (Fig. 6C) clearly showed that ablation of Bim expression protected cells from Con A–induced apoptosis. We also ablated Bim expression in SKOV3 cells and found that these cells were also protected from Con A after Bim knockdown (Fig. 6D).

**Discussion**

p53 plays a central role in the apoptotic response to chemotherapeutic agents. However, a great majority of human cancers either lack p53 or retain a mutant p53, which is functionally inactive. It is our understanding that an ideal compound that holds promise in cancer chemotherapy would possess two essential properties: (a) able to activate the apoptotic pathway in the absence of p53 and (b) able to activate p53, which in turn would protect normal cells by inducing a transient, reversible growth arrest in the presence of functional p53. In the present study, we have found that Con A possessed such properties. Con A treatment induced apoptosis of cells lacking functional p53. In contrast, rescue of p53 function in the same cells protects them from Con A–induced cytotoxicity by inducing a reversible growth arrest at the G1 phase of the cell cycle because most of these cells are capable of forming colonies after Con A withdrawal (28). Accumulating evidence suggests that Con A treatment causes conformational changes in the p53 protein by posttranslational modifications without changing p53 protein levels (32–34). However, the functional and/or biological consequence of this p53 modification by Con A was unknown. Consistent with these studies, we have also found that Con A treatment modulates the phosphorylation status of several serine residues of p53 without changing p53 protein levels. These changes in...
phosphorylation status transcriptionally activate p53, which in turn protects cells from Con A–induced apoptosis, because ablation of p53 by shRNA or pretreatment with PFT inhibits p21 expression. Moreover, p53 transcriptional activity is required for its protective effect against Con A.

The FOXO transcription factors are emerging as key regulators of cell survival. Activation of FOXO transcription factors induces apoptosis by upregulating several cell death genes, including those encoding the ligand for the death receptor known as Fas or CD95, Bim, and the tumor necrosis factor–related apoptosis-inducing ligand (35–37). On the other hand, overexpression of FOXO3a, a member of the FOXO family, can protect cells from oxidative stress–induced cell death (38, 39). Induction of several antioxidant enzymes and stress-related gene products has been proposed as a potential mechanism (35, 40, 41). The fact that the precise biological consequences of FOXO activation are cell type specific and stress type dependent suggests that there might be cross-talk between FOXO and other stress regulators. Studies thus far suggest striking similarities between the p53 family members and the FOXO family members: both have similar regulatory mechanisms and several common downstream target genes, and activation of both groups leads to cell cycle arrest or apoptosis, depending on the cellular context and type of stress. Although p53 shares parallel functions with FOXO transcription factors, our data clearly show a previously unidentified, Con A–dependent cross-talk between p53 and FOXO1a. Treatment of cells lacking p53 with Con A induced FOXO1a at both the mRNA and protein levels. Moreover, expression of its proapoptotic target, Bim, was found to increase at both the mRNA and protein levels, ablation of which protects cells from Con A cytotoxicity, suggesting FOXO1a–Bim–mediated apoptosis in the absence of p53. Interestingly, expression of p53 in these cells strongly inhibited the expression of FOXO1a and Bim at both the mRNA and protein levels and protected the cell from Con A–induced apoptosis, suggesting transrepression of FOXO1a and Bim by p53. The ability of p53 to transrepress certain genes is critical for some of its biological responses. However, the molecular mechanisms of p53-mediated transrepression remain a controversial area of p53 biology. Several mechanisms have been proposed, including interference with the function of transcriptional activators, interference with the basal transcriptional machinery, recruitment of chromatin-modifying factors to reduce promoter accessibility, and recruitment of transcriptional corepressors (42). Moreover, transcriptional repression of certain genes by p53 can also occur indirectly through the ability of p53 to transactivate p21 (43–45). Whether the transrepression of FOXO1a by p53 is mediated directly or indirectly via p21 is currently under investigation.

FOXO transcription factors are direct substrates of Akt. Akt–dependent phosphorylation of FOXO resulted...
in its cytoplasmic localization, thereby inhibiting FOXO-induced target gene expression (35). However, we found a variable effect of p53 expression on Akt activation after Con A treatment. Con A activates Akt in TR9-7 cells, but not in 041 cells, suggesting p53-dependent activation of Akt. In contrast, Con A activates Akt in SKOV3 cells, but not in SKP53 cells, suggesting p53-dependent inhibition of Akt activation (data not shown). Moreover, overexpression of constitutively active Akt in 041 cells fails to protect from Con A cytotoxicity. Together, these results suggest that transrepression rather than inhibition of posttranslational modification of FOXO1a is more important for p53-mediated inhibition of FOXO1a, and Akt activation is dispensable.

In conclusion, our study suggests that Con A has potential promise in the treatment of certain types of cancer because Con A is able to trigger p53-independent apoptosis of cells lacking p53, whereas those with p53 comparable with that in normal cells are protected.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. George R. Stark (Cleveland Clinic Foundation) for his continuous encouragement and criticism throughout the course of the work, Dr. Anthea Hammond (Emory University) for editorial assistance, and Dr. Rajib K. Paul for screening SKP53 cells.

**Grant Support**

NIH grant R01 CA98916 (M.L. Agarwal) and NIH grant P50 CA128613 (D.M. Shin and A.R.M.R. Amin).

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Received 8/7/09; revised 11/24/09; accepted 12/15/09; published OnlineFirst 2/2/10.

**References**


Molecular Cancer Therapeutics

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