A Novel Role for p73 in the Regulation of Akt-Foxo1a-Bim Signaling and Apoptosis Induced by the Plant Lectin, Concanavalin A

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Abstract

Virtually all human cancers encounter disruption of the “p53 network.” From a therapeutic point of view, it is important to devise strategies that eliminate cancer cells, which are often defective in functional p53 and protect p53-expressing normal cells. By comparing the response of a pair of isogenic cell lines, we identify a plant-derived compound, Concanavalin A (Con A), which differentially kills p53-null cells. Further, we find that p53 family member, p73, plays a critical role that is unmasked in the absence of p53. Con A treatment leads to induction of p73 and several others that are important mediators of apoptosis and act downstream, such as p21, Bax, Foxo1a, and Bim. Inactivation of p73 reverses the expression of these proteins and apoptosis. Inhibition of Akt activation sensitizes otherwise resistant cells. These observations thus reveal a novel role for p73 in the regulation of Akt-Foxo1a-Bim signaling and apoptosis especially when p53 is absent. [Cancer Res 2007;67(12):5617–21]

Introduction

In normal cells, the p53 tumor suppressor plays a pivotal role in regulating the cell cycle checkpoints, apoptosis, genomic integrity, and DNA repair in response to various forms of genotoxic stress (1, 2). Due to its role in maintaining genomic integrity and cell cycle checkpoints, p53 has been termed the “guardian of the genome.” Unfortunately, p53 is mutated in ~50% of human cancers and functionally inactivated in a further 20% (1–3). It is therefore important to devise therapeutic strategies that target cancer cells with defective p53 while protecting normal cells. To identify compounds that exhibit differential growth-inhibitory effects dependent on p53 status, we compared isogenic human fibroblasts lacking p53 with those reconstituted with a tetracycline-controlled dominant-negative p73 using Lipofectamine Plus reagent according to supplier’s instruction. Individual clones were selected using G418.

Materials and Methods

Cell culture, plasmid transfection, and cell treatment. All cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin in a CO2 incubator. For Con A treatment, 3 × 10^4 cells were plated in a 10-cm culture dish. After overnight incubation, the old media were replaced with fresh one and the cells were treated with 15 μg/ml Con A for the indicated times. 041 cells were transfected with dominant-negative p73 using Lipofectamine Plus reagent according to supplier’s instruction. Individual clones were selected using G418.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. For apoptosis assay, cells were grown in 10-cm culture dishes and treated with 15 μg/ml Con A for 72 h. The cells were fixed in 70% ethanol and labeled with fluorescein-tagged bromo-dUTP and propidium iodide as per manufacturer’s protocol using APO-bromodeoxyuridine apoptosis kit (Phoenix Flow Systems).

RNA isolation and real-time PCR. Total RNA was extracted from the cells by using 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.

Western blot analyses. SDS-PAGE and immunoblotting were done as described elsewhere (7). Briefly, 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 with the respective antibody followed by the incubation with a secondary antibody. Proteins were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) as directed by the manufacturer.

Results

Con A treatment selectively induces apoptosis in cells lacking p53. To identify compounds that exhibit differential effects on cellular growth in the presence or absence of p53, we made use of an isogenic pair of human fibroblasts, p53-null MDAH041 (041), its derivative, TR9-7–expressing p53 (10), and ovarian cancer cell SKOV3 that lacks p53. After a long screening process, Con A was found to exhibit profound differential apoptosis in cells lacking p53. 041 and SKOV3 cells became rounded and detached, whereas a large population of TR9-7 cells remained adhered with normal morphology. Methylene blue staining of the
plates showed a profound differential growth regulation depending on the p53 status (Fig. 1A). Next, a colony-forming assay was done to determine whether the treated cells could resume growth. After 7 days of treatment with Con A, 500 live cells were plated onto a 15-cm culture plate in the absence of Con A. After 3 wks, the colonies formed in each plate were counted after methylene blue staining. Results are expressed as percentage of untreated cells. C, 041, TR9-7, and BJ cells (normal human fibroblast) and SKOV3 cells were treated with 15 μg/mL Con A for 72 h. Cells were fixed in 70% ethanol and stained for TUNEL-positive cells. D, cells were treated with 15 μg/mL Con A for 24 h. Total cell lysates were subjected to Western blotting for Bax, Bcl2, or PARP.

Figure 1. Con A induces apoptosis of cells lacking p53. A, cells were treated with 15 μg/mL Con A for 72 h and stained with methylene blue. B, cells were treated with 15 μg/mL Con A for 7 d. The adhered cells were trypsinized and 500 live cells were plated in 15-cm culture plates in the absence of Con A. After 3 wks, the colonies formed in each plate were counted after methylene blue staining. Results are expressed as percentage of untreated cells. C, 041, TR9-7, and BJ cells (normal human fibroblast) and SKOV3 cells were treated with 15 μg/mL Con A for 72 h. Cells were fixed in 70% ethanol and stained for TUNEL-positive cells. D, cells were treated with 15 μg/mL Con A for 24 h. Total cell lysates were subjected to Western blotting for Bax, Bcl2, or PARP.

Poly(ADP-ribose) polymerase degradation and modulation of Bax/Bcl2 ratio in favor of apoptosis. To investigate the mechanism of Con A–induced apoptosis, we next examined the expression of proapoptotic protein Bax and antiapoptotic protein Bcl2 and the degradation of poly(ADP-ribose) polymerase (PARP). Western blot analysis, as shown in Fig. 1D, reveals that degradation of PARP was observed only in 041 cells. The levels of Bax increased much more in 041 cells than in TR9-7 cells. Furthermore, Bcl2 expression levels were drastically decreased only in 041 cells. Thus, Con A treatment modulated the Bax/Bcl2 ratio in favor of apoptosis much more in 041 cells than in TR9-7 cells.

Con A–induced apoptosis is mediated via p73 in cells lacking p53. To investigate the mechanism of apoptosis in cells lacking p53, we analyzed the expression of p73, a member of the p53 family of transcription factors. Western blot analysis and real-time PCR, as shown in Fig. 2A, reveals that Con A treatment induced the expression of p73 in both 041 and SKOV3 cells. To further implicate the role of p73 in Con A–induced apoptosis, p73 was functionally inactivated by using a dominant-negative mutant of p73 (11). 041 cells were transfected with a T7-tagged DNp73. Two independent clones C12 and C17 expressing DNp73 were established (Fig. 2B). Con A–treated cells from these two clones were stained for TUNEL to measure apoptosis and lysates were analyzed by Western blot for the expression of Bax and Bcl2. As shown in Fig. 2B and C, expression of dominant-negative p73 rendered resistance to Con A–induced apoptosis as revealed by decrease in TUNEL-positive cells and reversal of the expression of both Bax and Bcl2 proteins compared with that in parental 041 cells. Real-time PCR analysis also showed that the expression of p21 and Bax is p73 dependent (Fig. 2D). These data together indicate that p73 protein mediates apoptosis in response to Con A treatment and that the expression of some of the p53 target genes is regulated by p73 in the absence of p53.
p73-dependent expression of Foxo1a and Bim. To identify molecular components involved in Con A–induced apoptosis, we did microarray analysis. Total RNA from 041 cells either untreated or Con A treated were isolated and hybridized with a chip containing ~26,000 transcripts from human origin. Expression pattern of a total of 1,151 genes was found to be modulated (2-fold change). A careful analysis of the data revealed that the forkhead transcription factor Foxo1a and its proapoptotic target Bim (also known as Bcl2L11) were well induced after Con A treatment. We further confirmed the microarray data by Western blot analysis. As shown in Fig. 3, Con A treatment induced a profound increase in the level of both Foxo1a and Bim protein. Because both Foxo1a and Bim are involved in apoptosis and Con A–induced apoptosis is mediated via p73, we next examined the expression of Foxo1a and Bim in cells expressing dominant-negative p73. As shown in Fig. 3B and C, Con A treatment led to an increase in the expression of Foxo1a and Bim at both mRNA and protein levels in parental 041 cells. In contrast, their expression levels were substantially decreased in cells with inactivated p73. We also measured the expression of Bim in these cells after treatment with a known DNA-damaging agent, camptothecin. Treatment with camptothecin also substantially increased the expression of Bim. However, there was no difference between the cell lines (data not shown). These results suggest that expressions of both Foxo1a and Bim are regulated by p73 in response to Con A.

p73-dependent inhibition of Akt activation is critical for Con A–induced apoptosis. The serine/threonine kinase Akt plays a pivotal role in the regulation of Foxo transcription factors, which are the direct substrates for Akt (12). Because Con A treatment induced the expression of Foxo1a and its target Bim, we next examined the activation of Akt in 041 cells and its derivative clones expressing DNp73. As shown in Fig. 4A, there was very little phosphorylation of Akt in 041 cells. In contrast, Con A–mediated phosphorylation of Akt was greatly induced in the same very cells but expressing DNp73. These results suggest that activation of p73 by Con A inhibits the phosphorylation of Akt. To further confirm the role of p73-dependent inhibition of Akt activation in Con A–induced apoptosis, cells expressing DNp73 (C17) were pretreated with phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, followed by treatment with Con A. As shown in Fig. 4B and C, pretreatment with LY294002 completely inhibited Akt activation and sensitized cells to Con A–induced apoptosis. These results suggest that p73-dependent inhibition of Akt activation is important for Con A–induced apoptosis.

Discussion

A great majority of human cancers either lack p53 or retain a mutant p53 that is functionally inactive. An ideal compound for cancer chemotherapy would posses the ability to activate apoptotic pathways in tumor cells lacking p53 and simultaneously protect normal cells retaining p53. In the present study, we find that Con A possesses such properties. Cells lacking functional p53 undergo apoptosis. In contrast, the same cells expressing p53, as well as normal human fibroblasts, are much less sensitive to Con A and resumed growth upon return to their normal conditions. In the present report, we have investigated the molecular mechanism of apoptosis in response to Con A in the absence of p53. Unlike p53, its family member p73 is frequently functional and is overexpressed in some tumors (13). Therefore, p73 is now regarded as an important determinant of cellular sensitivity to anticancer drugs, particularly in tumors lacking functional p53 (reviewed in refs. 3 and 14). These findings have aroused interest in searching for compounds that can activate p73 and induce p73-dependent apoptosis in cancer cells lacking functional p53. We found for the first time that Con A–induced apoptosis is p73 dependent because inhibition of p73 function by a dominant-negative construct almost completely reversed the Con A response. Our observations show that Con A induces p73, which in turn modulates the Bax/Bcl2 ratio such that it is more favorable to apoptosis. Many reports
suggest that activation of p73 is dependent on E2F1. In contrast, we observed recently that the induction of p73 is dependent on reactive oxygen species (ROS) generation in response to (-)-epigallocatechin-3-gallate (15). However, we found that the expression of E2F1 was drastically decreased in 041 and its derivative clones, but ROS generation was substantially increased after Con A treatment (data not shown). These results suggest that Con A–induced expression of p73 is independent on E2F1 but might be regulated by ROS.

The Foxo family of transcription factors, including Foxo1a and Foxo3a, are emerging as key regulators for cell survival and apoptosis. Activation of Foxo induces apoptosis by up-regulating several cell death genes, including those encoding the ligand for the death receptor known as Fas or CD95, the Bcl2-interacting mediator (Bim) of cell death, and the tumor necrosis factor–related apoptosis-inducing ligand (12, 16, 17). However, the mechanism of induction of Foxo is largely unknown. In the present study, we for the first time showed that the plant lectin Con A induced the expression of both Foxo1a and its proapoptotic target Bim at the transcriptional and protein levels in cells undergoing apoptosis. Moreover, our results also revealed for the first time that p73 plays a critical role in the regulation of these genes.

The Akt serine/threonine kinases are critical mediators of cell survival in response to many extracellular stimuli. It is now clear that the activation of the PI3K-Akt pathways and the resulting inhibition of apoptosis are critical events in tumorigenesis. Several Akt downstream targets are important for the survival role of Akt, including Foxo1a, Bad, MDM2, p21, etc. (18). Our results suggest that activation of p73 inhibits the activation of Akt following Con A treatment, which was rescued after disruption of p73 activation and which mediates the apoptosis in cells lacking p53. To our knowledge, this is the first report that describes p73-dependent inhibition of Akt activation to mediate apoptosis. The Foxo transcription factors, including Foxo1a, are direct substrates for Akt. Phosphorylation of Foxo1a by Akt exports it from the nucleus to the cytoplasm, thereby inhibiting Foxo1a-induced target gene expression and apoptosis (12). There is insignificant activation of Akt in 041 cells; thus, Foxo1a remains fully active and induces plenty of Bim to execute apoptosis. In contrast, rescue of Akt phosphorylation in cells with disrupted p73 activation might inactive Foxo1a by exporting it from the nucleus in addition to inhibition of Foxo1a transcripts, thus inhibiting Bim expression and complete reversion of apoptosis.

The cyclin-dependent kinase (CDK) inhibitor p21 is a bona fide target of p53 family members and is involved in both G1 and G2-M arrest to allow time to repair any sort of genomic damage caused by genotoxic stress. Consistent with the study by Desrivieres et al. (19), we also found that Con A treatment induced both G2-M and G1 cell cycle arrest (data not shown). However, this is the first report to show that Con A induced p21, which is mostly dependent on p73. A recent study by Huang et al. (20) suggests that CDK2 phosphorylates Foxo1a and

![Figure 3. p73-dependent expression of Foxo1a and Bim. A, 041 cells were treated with Con A for the times indicated and total cell lysates were analyzed by Western blotting using antibodies that detect Foxo1a and Bim. B, 041 and its derived cells expressing DNp73 were treated with Con A for times indicated and cellular lysates were analyzed by Western blotting using Foxo1a and Bim antibodies. C, cells were treated with Con A for 24 h and total RNA was isolated and subjected to real-time PCR for Foxo1a and Bim.](image)

![Figure 4. Inhibition of Akt activation by p73 in Con A–induced apoptosis. A, cells were serum starved overnight and treated with 15 μg/mL Con A for 30 min. Total cell lysates were analyzed by Western blotting against anti–phospho Akt (Ser473). B, overnight serum starved 041 cells expressing dominant-negative p73 (C17) were pretreated with 20 μmol/L LY294002 (LY) for 1 h followed by stimulation with 15 μg/mL Con A for 30 min. Total cell lysates were analyzed by Western blotting against anti–phospho Akt. C, C17 cells were pretreated with LY294002 for 1 h followed by Con A treatment for 72 h. Cells were stained for TUNEL-positive cells.](image)
renders it transcriptionally inactive. Activation of checkpoint pathways on DNA damage abrogated this phosphorylation of Foxo1a to execute apoptosis. Because p21 is an inhibitor of CDK2, expression of p21 by Con A treatment might also cooperate to transactivate Foxo1a to induce apoptosis. In conclusion, our study suggests a novel role for p73 in the regulation of Akt-Foxo1a-Bim signaling and apoptosis. Furthermore, these cell culture studies also suggest that Con A exhibits properties that would make a logical sense for a chemotherapeutic agent: to be able to trigger apoptosis in cells lacking functional p53 (e.g., cancer cells) and inducing a transient arrest and thus protective effect of p53-expressing normal cells.

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References

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