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News and Commentary

p53 downstream targets and chemosensitivity

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Introduction

The product of the tumor suppressor gene, p53, is activated by a variety of cellular responses including DNA damage, oncogene stimulation, and nucleotide depletion.[1] The most well-understood role of p53 is its ability to regulate the transcription of target genes involved in cell cycle arrest, senescence, and programmed cell death.[1] Transient or irreversible p53-mediated cell cycle arrest in the transition from G1 to S phase is mediated by the transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$.[2,3] GADD45 and 14-3-3σ are also transcriptionally activated by p53 for the maintenance of a G2 arrest.[4,5] In recent years, a number of p53 target genes have been identified that mediate apoptosis; however, no single target gene has been shown to be required for the full response. In addition, the repression of some target genes by p53 may also be important for initiating programmed cell death.[6,7]

Molecules involved with both the death receptor- and mitochondria-mediated apoptotic pathways are regulated by p53 in response to cellular stress.[8] The death receptor pathway is initiated by the formation of the death inducing signaling complex (DISC) at the cell membrane. The combination of receptor aggregation, association of adaptor proteins, such as FADD, and the oligomerization and autocatalyzation of initiator caspase -8 and -10 leads to the processing of downstream effecter caspases and the cleavage of molecules resulting in apoptosis.[9] In certain cell types where the formation of the DISC is not sufficient to lead to apoptosis, the mitochondria-mediated apoptotic pathway is employed.[9] Members of the Bcl-2 family with both pro- and antisurvival functions interact with each other. When the balance between the pro- and anti-survival Bcl-2 family members shifts towards apoptosis, the mitochondria membrane potential is disrupted and molecules, such as cytochrome *c* and Smac/Diablo, are released into the cytosol and can then lead to the activation of effecter caspases involved in carrying out apoptosis.[10]

Stabilized p53 can initiate the transcription of genes involved in cell cycle checkpoints and in both death pathways.[11] While the induction of some genes may be enough to initiate cell death, the induction of other genes by themselves will not cause the cell to undergo apoptosis. A new class of p53 target genes is emerging (Figure 1), where the induction of the genes is important to sensitize cells to chemotherapeutic agents, but by themselves the expressed genes are not lethal to the cell. In this way, the cell requires both the induction of the gene and additional stimuli to initiate the death pathway.

p53 Targets in Cell Cycle Checkpoints

Treatment of cells with DNA-damaging agents induces both a G1 and G2 cell cycle arrest and this effect is, for the most part, dependent on the presence of wild-type p53.[1] Cell cycle arrest following exposure to chemotherapeutics or ionizing radiation is considered to be a major way by which p53 suppresses tumor formation.[1,12]

The identification of p53 targets involved in p53-mediated cell cycle arrest has shown that the CDK inhibitor $p21^{WAF1/CIP1}$ is required for the induction of a G1 arrest.[13–15] However, the generation of p21-deficient mice did not result in the same phenotype as p53-deficient mice suggesting that p21 is not the only p53 target gene required for p53-mediated tumor suppression.[15] In addition, GADD45 and 14-3-3σ appear to regulate G2/M progression in response to ionizing radiation. Induction of GADD45 is dependent on wild-type p53 and the ATM/ATR kinase that is defective in ataxia telangiectasia patients, suggesting that this gene may be an important target for tumor suppression.[5] 14-3-3σ is involved in regulating G2/M progression in response to ionizing radiation.[4] Taken together, p53 transcriptionally activates genes involved in preventing both G1/S and G2/M progression and these genes are important for tumor suppression; however, they do not comprise the whole story, p53 also transcriptionally targets genes involved in apoptosis.

p53 Targets in the Extrinsic Pathway

Binding of death ligands to death receptors at the cell membrane activates the apoptotic process. This pathway is

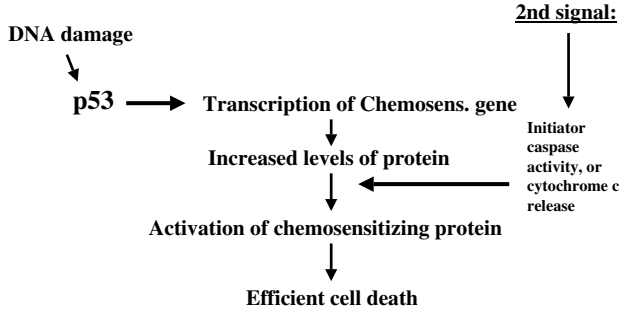

Figure 1 p53 dependent transactivation of "chemosensitivity genes" lowers apoptotic threshold

tightly regulated both by death inhibitors and composition of molecules at the cell membrane within the cell. Members of the tumor necrosis factor receptor (TNFR) superfamily may initiate either a survival or cell death cascade depending on the cellular context.[16] For the purposes of this review, we will focus on the cell death pathway.

Two proapoptotic members of the TNFR superfamily, Fas/Apo1 and Killer/DR5, are regulated in a p53-dependent manner in response to multiple chemotherapeutic drugs.[17–19] In some cases, the upregulation of the death receptors alone is sufficient to initiate the cell death cascade. The regulation of the extrinsic pathway at essentially the most upstream cellular level, that is, at the plasma membrane, provides the link between DNA damage and the initiation of a cascade that can work either directly to induce apoptosis or to enlist the help of the mitochondria.

An additional membrane-bound protein named p53 apoptosis effector related to PMP-22 (PERP) was identified as a p53 target gene that induces apoptosis; however, the precise mechanism by which this occurs is not fully understood.[20] Possibly localized to the plasma membrane, endoplasmic reticulum, mitochondria, and golgi apparatus, overexpression of PERP is able to induce cell death that can be blocked by the overexpression of Bcl-2. Although the PERP pathway to cell death is unclear, it is clear that this p53 transcriptionally induced membrane-bound protein is able to initiate an apoptotic pathway.[20]

p53-induced protein with a death domain (PIDD) was recently identified as a p53-regulated cytoplasmic protein containing a death domain.[21] PIDD mRNA can be induced by double-stranded breaks in DNA caused by ionizing radiation. Moreover, the basal level of PIDD expression appears to be dependent on p53 status as p53-deficient marine embryonic fibroblasts (MEFs) have much lower expression of PIDD compared to wild-type MEFs. Overexpression of PIDD inhibits cell growth and antisense ablation of PIDD attenuates p53-mediated cell death.[21] However, the precise mechanism by which PIDD induces apoptosis remains to be elucidated.

p53 Targets in the Intrinsic pathway

Mitochondrial dysfunction initiates the intrinsic apoptotic pathway. This pathway is also tightly regulated both by death inhibitors and the balance between prosurvival and proapoptotic Bcl-2 family members.[10] Recent evidence suggests that p53 requires apoptotic protease activating factor 1 (Apaf-1), caspase-9, and cytochrome *c* release to carry out apoptosis.[22] Hence, the intrinsic pathway appears to be vital for p53-dependent apoptosis and tumor suppression.[22]

Perhaps, the founding member of the p53 target genes involved with mitochondrial-mediated apoptosis is Bax. Bax is a proapoptotic member of the Bcl-2 family. Binding of Bax to the antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, causes a shift in the tug-of-war from survival towards apoptosis.[10,23] Moreover, it appears that p53 can decrease the expression of Bcl-2; however, the mechanism behind the repression remains unclear.[24]

Over the past few years, several p53 target genes have been identified that are proapoptotic mediators of mitochondrial dysfunction and subsequent cell death including two proapoptotic Bcl-2 family members, PUMA (p53 upregulated modulator of apoptosis) and Noxa. PUMA is a BH-3-only member of the Bcl-2 family that can interact with both Bcl-2 and Bcl-X_L and initiate cell death. Overexpression of PUMA suppresses colony formation in human tumor cell lines presumably mediated by PUMA-mediated apoptosis, and the inhibition of PUMA expression by antisense strategy attenuates the p53-mediated apoptotic response.[25,26] Noxa, another BH-3-only member of the proapoptotic Bcl-2 family, is regulated by p53. Like other proapoptotic Bcl-2 family members, Noxa is able to interact with pro-survival Bcl-2 family members to initiate mitochondria permeability changes.[27]

In response to DNA damage, p53 is phosphorylated on serine-46 and this phosphorylation event appears to be required for the induction of p53-regulated apoptosis-inducing factor (p53AIP1).[28] p53AIP1 is localized to the mitochondria. Although p53AIP1 is not a member of the Bcl-2 family, it appears to be able to initiate mitochondria membrane dysfunction leading to apoptosis.[28]

While the p53 targets described above can be compartmentalized into either cell cycle progression, death receptor pathway, or mitochondria pathway, a number of p53-induced genes appear to be induced, prior to the generation of reactive oxygen species (ROS) and these genes encode cytoplasmic proteins that ultimately signal through mitochondria-mediated cell death.[29,30] The p53-induced genes (PIGs) were initially isolated using the SAGE technique following p53 overexpression.[29] In this screen, PIG8 turned out to be the human homologue of murine EI24 (etoposide induced 24), a previously identified p53 target gene that when overexpressed has been shown to suppress colony formation and induce apoptosis.[31,32] Many of the identified PIG genes are predicted to encode proteins that could generate or respond to ROS. Indeed, additional characterization provided a potential framework for the induction of these genes by p53. p53 can transcriptionally activate a subset of genes (i.e. PIGs) involved in the production of ROS, which in turn damage the mitochondria and induce apoptosis, hence contributing to tumor suppression.[29]

p53 Targets and Chemosensitivity

While the induction of some p53 target genes appears to be sufficient to initiate apoptosis, the induction of other target genes either does not induce apoptosis or induces apoptosis to low levels without additional stimulation or processing. Recent data suggest a new class of p53 target genes that sensitize cells to the effects of chemotherapeutic agents (Figure 1).

Although not initially described as a p53-chemosensitization gene, Apaf-1 may be the founding member of this new classification. Apaf-1 was originally identified as the human homologue to the *Caenorhabditis elegans* CED-4, with additional sequence similarity to CED-3 (both CED-3 and CED-4 are required for apoptosis in *C. elegans*).[33] *In vitro* reconstitution of caspase-3 activation showed that addition of Apaf-1 alone does not appear to initiate caspase-3 processing. However, addition of Apaf-1, in the presence of excess dATP, cytochrome *c*, and Apaf-3 is then able to activate caspase-3.[33] Indeed, recent data has shown that

cytochrome *c*, Apaf-1, and caspase-9 form a ternary complex, requiring dATP, named the apoptosome.[34] Once formed, the apoptosome is able to process caspase-9 which in turn activates caspase-3.[34] The generation of Apaf-1 knockout mice have shown that Apaf-1 is required for the activation of caspase-3 in the brain and as such, Apaf-1$-/-$ mice are embryonic lethal because of the developmental defects.[35,36] However, one study did obtain 3 Apaf-1$-/-$ mice that lived to day 10 and these mice provided valuable information.[36] Thymocytes from the knockout mice were resistant to various forms of DNA damage including dexamethasone, etoposide, and γ-irradiation, while the thymocytes remained sensitive to Fas-mediated cell death. Moreover, activated peripheral T cells from Apaf1$-/-$ mice were resistant to UV treatment but remained sensitive to the apoptotic effects of the Fas ligand. These data suggested that Apaf-1 is important for mitochondrial-mediated apoptotic death, but not required for death receptor-mediated cell death.[36]

Recent evidence has shown that Apaf-1 is a target of both E2F and p53.[37] Either the pRb or the p53 pathways are deregulated in most types of tumors. Combining *in vitro* evidence that Apaf-1 is not a potent apoptosis-inducing gene by itself without the help of additional factors released from the mitochondria and the evidence that Apaf-1 is transcriptionally regulated by E2F and p53 suggests that the transcriptional regulation in combination with other signals that mediate mitochondrial membrane potential dysfunction, such as signals caused by chemotherapeutic agents, allows for the release of cytochrome *c* that can then work synergistically with the increased Apaf-1 levels to initiate apoptosis. Hence, the induction of Apaf-1 would appear to sensitize the cells to apoptosis induced by additional signals.

More recently, the executioner caspase-6 was identified as a p53 transcriptional target gene.[38] The caspase family are cysteine-directed proteases that normally exist in a latent (pro-) form, but when activated, by cleavage of the prodomain, are potent inducers of cell death. Caspases are divided into two categories, the initiator caspases (-8, -10, -9, -2, -12) are activated upon death stimuli that then activate the effecter caspases (-3, -6, -7) that then cleave critical substrates important for cell survival.[39,40] Hence, the executioner (effecter) caspases represent one of the most downstream components of apoptosis.

Caspase-6 mRNA and protein levels were induced following overexpression of p53 and treatment of cells with adriamycin. While the overexpression of p53 or the exposure of cells to adriamycin led to some caspase-6 activity, the combination of the two induced significantly more caspase-6 activity that was not because of increased levels of mRNA or protein as observed with overexpressed p53 alone. It appears that more procaspase-6 protein was cleaved to the active form in the presence of both p53 and adriamycin, suggesting that while p53 induces procaspase-6, additional signals are required to initiate the activation of this potent enzyme. Therefore, the induction of caspase-6 by p53 may lower the cell's threshold to the effects of chemotherapeutic agents.[38]

The third member of this emerging class of chemosensitization targets is the proapoptotic Bcl-2 family member Bid.[41] Bid is a cytoplasmic protein that is cleaved within an unstructured loop by active caspase-8. The cleavage exposes a new N-terminal glycine that is then post-transcriptionally myristoylated. The truncated Bid (tBid) then translocates to the mitochondria and inserts into the membrane.[42] In combination with two other proapoptotic Bcl-2 family members, Bax and Bak, these proteins initiate mitochondrial dysfunction and ultimately apoptosis.[43,44] Bid is the link between activation of the extrinsic and intrinsic pathways in cells that require mitochondria events to amplify the cell death signal.[45]

Bid was initially identified as a p53 transcriptional target in an Affymetrix screen using the p53-expressing temperature-sensitive cell line Vm10. Bid mRNA levels were induced both *in vitro* and *in vivo* by p53. Interestingly, Bid-deficient mouse embryonic fibroblasts (MEFs) treated with increasing doses of adriamycin or 5-fluorouracil were significantly more resistant to apoptosis than wild-type MEFs, suggesting that Bid is required for an effective chemotherapeutic effect, at least in MEFs. These results suggest that while the induction of full-length Bid alone is not able to induce cell death, additional stimuli will initiate the processing of Bid and initiate apoptotic death.[41] Therefore, the induction of Bid by p53 helps to sensitize the cells to the toxic effects of chemotherapeutic drugs.

p53 Targets and Genomics

Recent technical advances have opened up new avenues to identify novel p53 target genes and hopefully help elucidate why p53 regulates so many genes. The combination of the sequencing of the human genome and increasing computational resources has been applied to identify p53-regulated genes.

DNA microarray technology has allowed for high-throughput analysis of genes induced by p53 under a variety of conditions.[46] Indeed, numerous p53 target genes have been identified using this technique. On a larger scale, the arrays can be used to compare which p53 target genes are induced following different treatments, and newly induced genes that have not yet been characterized can be grouped, based on sequence, to different compartments of the cell.[46] In fact, there appears to be endless ways to characterize, group, and analyze these microarray screens. The microarray screens provide a powerful tool to identify novel genes, and the understanding and characterization of these genes will help to elucidate the complexity of p53-mediated pathways.

A recent study used a computer algorithm to identify novel p53-regulated genes by scanning the genome for p53-binding sites.[47] p53-binds to a specific sequence comprised of two decamers separated by 0–13 bp.[48] The computer algorithm identifies potential p53-binding sites by a scoring system based on the percentage of similarity to a consensus sequence. Approximately 4000 genes, separated into 14 biological categories were classified in this screen and can be found on a comprehensive website, http://linkage.rockefeller.edu/p53.[47]

As additional studies combine the available sequence information and high-throughput screening tools, we will hopefully be able to combine, connect, and understand the complex role p53 plays in tumor suppression.

Conclusions

While over the past few years it has become clear that p53 regulates multiple genes involved in carrying out apoptosis, it has not been clear why so many different genes are induced in different cell types or under different conditions. Perhaps, the apoptotic pathway is so complex that p53 must be able to mix and match different target genes in order to get the desired effect. It has become increasingly clear over the past few years that p53 regulates genes involved in apoptosis in both the death receptor- and mitochondrial pathways in order to control cell survival.[30] In an attempt to understand why so many p53 target genes exist, recent evidence has shown distinct tissue specificity of various p53-regulated genes.[49,50] The tissue-specific distribution of p53-regulated genes and an increasing understanding of the function of each new target may begin to help elucidate the reason for such a large spectrum of gene induction. This new paradigm of chemosensitization genes may explain, in part, why p53 has such a large arsenal.

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