Biochemical and Biophysical Research Communications **265,** 1–6 (1999)
Article ID bbrc.1999.1446, available online at http://www.idealibrary.com on IDEAL®

BREAKTHROUGHS AND VIEWS

p53-Induced Apoptosis as a Safeguard against Cancer

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Received August 23, 1999

p53 acts as a potent tumor suppressor largely through its ability to induce cell death by apoptosis. Diverse cellular stress conditions, e.g., DNA damage, hypoxia, and oncogene activation, trigger p53-dependent apoptosis. ARF is a 14-kDa protein encoded by an alternative reading frame within the human INK4a locus that also encodes the p16 protein. ARF induces p53 in response to oncogene activation by preventing its degradation. This ensures the elimination of emerging tumor cells by p53-dependent apoptosis. p53 promotes apoptosis through multiple mechanisms, including transactivation of specific target genes, down-regulation of a distinct set of genes, and transcription-independent mechanisms. This may explain the frequent inactivation of ARF/p53 rather than downstream effectors during tumor development.

Tumors develop as result of multiple genetic changes and acquire a variety of new phenotypic characteristics. A common feature of tumor cells is loss of normal cell cycle control. Perhaps all tumor cells show dysfunction of the p16-cyclin D-cdk4-pRb pathway that regulates G1/S transition in the cell cycle (1). Another frequent target for mutations in tumors is the p53 gene. The fact that around half of all human tumors carry mutations in this gene is solid testimony as to its critical role as tumor suppressor. p53 halts the cell cycle and/or triggers apoptosis in response to various stress stimuli including DNA damage, ribonucleotide depletion, hypoxia, oxidative stress, and oncogene activation (2, 3) (Fig. 1). Upon activation, p53 initiates the p53-dependent biological responses through transcriptional transactivation of specific target genes carrying p53 DNA binding motifs. In addition, the multifaceted p53 protein may promote apoptosis through transcriptional repression of certain genes lacking consensus p53 binding sites, and transcription-independent mechanisms as well (4). Analyses of a large number of mutant p53 genes in human tumors have revealed a strong selection for mutations in the specific DNA-binding core domain of p53 (residues 94–292) (2, 5), strongly suggesting that specific DNA binding is important for p53-mediated tumor suppression.

Both p53-induced cell cycle arrest and apoptosis are probably involved in p53-mediated tumor suppression. While p53-induced cell cycle arrest may be transient, p53-induced cell death is irreversible and therefore presumably more potent to prevent tumor growth. There is indeed evidence from mouse tumor models (6) and human tumors (7) demonstrating that p53-dependent apoptosis can suppress tumor development *in vivo*, particularly in response to oncogenic signalling. Further studies of stress signalling to p53 and p53-dependent tumor suppression may eventually lead to more efficient cancer therapy. This review will focus on recent advances in the understanding of p53 activation by oncogenes and p53-induced apoptosis.

ONCOGENIC SIGNALLING TO p53

Overexpression of the c-myc and adenovirus E1A oncogenes have been shown to induce accumulation of p53 and p53-dependent apoptosis (8–11). The E2F1 gene that drives S phase entry and progression can also trigger p53-dependent apoptosis (12, 13). Oncogenic ras triggers p53 accumulation and G1 arrest phenotypically similar to senescence in primary cells (14). Thus, p53 can accumulate and induce cell cycle arrest and/or cell death in response to certain activated oncogenes and cell cycle control genes.

The discovery of an alternative reading frame within the INK4a locus and studies of its protein product (1, 15, 16) have provided new and important clues as to how such genes may signal to p53. This reading frame encodes a 19 kD protein in the mouse denoted p19ARF (for Alternative Reading Frame) and a smaller 14 kD protein, p14ARF, in human cells. ARF induces cell

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0006-291X/99 $30.00

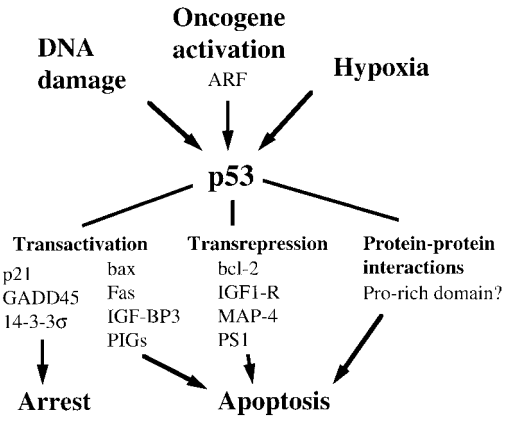

FIG. 1. A range of cellular stress signals induces stabilization and accumulation of the p53 protein. This may also involve conformational activation of p53. Oncogene activation induces p53 via the ARF protein that blocks MDM2-mediated p53 degradation, and possibly via other pathways as well. Induction of p53 triggers cell cycle arrest and/or apoptosis through transactivation of specific target genes, transrepression of other genes, and transcription-independent mechanisms, e.g., protein–protein interactions mediated by a proline-rich domain in p53.

cycle arrest in a strictly p53-dependent manner (15, 17). At least one important function of ARF is induction of p53 protein levels. Several studies showed that ARF binds to and inhibits the MDM2 protein that normally targets p53 for proteasome-mediated degradation, and thus stabilizes p53 (18–21). Recent work has painted a more complex picture of the interaction of ARF with MDM2 and p53, suggesting that ARF can block MDM2 function in several ways. ARF was shown to inhibit the ubiquitin ligase activity of MDM2 for p53, thereby stabilizing p53 (22). Other studies demonstrated that mouse ARF sequesters MDM2 in nucleoli, preventing MDM2-mediated nuclear export of p53 and degradation of p53 in cytoplasmic proteasomes (23, 24). Zhang and Xiong (25) found that human ARF leaves nucleoli to form nuclear structures with MDM2 and p53, blocking MDM2/p53 nuclear export. Tumor-associated mutations in ARF exon 2 were shown to abolish nucleolar localization of ARF and p53 stabilization. Therefore, the nucleolar localization of ARF appears important but its exact role remains unclear. An interesting question is whether nucleoplasmic ARF forms ternary complexes with MDM2 and p53 and whether such complexes, perhaps containing additional proteins, have any functional significance. Moreover, does ARF trigger conformational activation of p53?

ARF is induced by enforced expression of E1A, c-myc, and E2F1 (26–28). Oncogenic Ras also induces ARF according to one report (29), but others have obtained conflicting results (28). Induction of ARF probably involves both transcriptional and posttranscriptional mechanisms, including direct transcriptional transactivation of the ARF promoter by E2F1 (26, 28, 30). Both c-myc and E2F1 can induce p53 in ARF null cells, demonstrating that p53 accumulation caused by these proteins is not entirely ARF dependent (28). Likewise, loss of pRb can induce p53-dependent apoptosis, probably due to E2F1 activation. pRb knock-out mice exhibited p53-dependent apoptosis in the eye lens, which was abolished by crosses into a p53 null background (31). Importantly, lens fiber cell apoptosis was attenuated in mice null for both pRb and INK4a/ARF, consistent with the idea that ARF is required for the p53-dependent apoptotic response to loss of pRb (19).

Disruption of ARF/p53-dependent apoptosis appears essential for immortalization of mouse cells. Constitutive c-myc expression drives inactivation of either ARF or p53 by apoptotic selection in mouse embryo fibroblasts, resulting in apoptosis-resistant clones (28). Similarly, v-abl oncogene-mediated induction of ARF was shown to select for p53 mutation during Abelson murine leukemia virus-mediated pre-B cell transformation (32). ARF expression is increased in presenescent mouse fibroblasts (23), consistent with the possibility that ARF has a role in cellular senescence. However, ARF is not induced in senescing human keratinocytes (33), nor fibroblasts (34). This may reflect fundamental differences in ARF function and control of cellular life span between mouse and human cells. A final conclusion must await further studies of ARF expression in presenescent and senescent human cells of various types.

Regardless of its role in senescence, available evidence suggests that ARF acts as a cellular sensor for illegitimate growth signals and elicits p53-dependent apoptosis as part of a tumor surveillance program. In agreement with this notion, ARF null mice, like p53 null mice, develop tumors with a high incidence (35, 17, 36). However, tumors appeared later in ARF null mice than in p53 null mice, presumably because ARF is mainly activated by oncogenic signalling (as far as is known) whereas p53 responds to a diversity of stress signals including hypoxia and various types of DNA damage. Additionally, a wider tumor spectrum was observed in ARF null mice, possibly due to their longer survival compared to p53 null mice (36). The role of ARF as regulator of p53-triggered apoptosis in response to oncogene activation in human cells needs further study. Nonetheless, the frequent genetic lesions in the INK4a/ARF locus in human tumors imply that human ARF is a significant tumor suppressor.

MECHANISMS OF p53-INDUCED APOPTOSIS

In addition to the multiplicity of p53-activating signals, multiple mechanisms contribute to the p53-dependent biological response. The sequence-specific transactivation (SST) function of p53 is required for p53-induced cell cycle arrest and apoptosis in most experimental systems and cosegregates with p53:s tumor suppressor activity (37). The p21, GADD45,

14-3-3σ and other gene products are downstream effectors of p53-induced cell cycle arrest (38) (Fig. 1). p53-dependent apoptosis appears to involve transactivation of an entire set of target genes. The current catalog of candidate apoptosis-promoting p53 target genes includes among other genes bax, Fas, DR5/KILLER, IGF-BP3, and several PIG genes (38). Further work will surely add more genes to this category.

p53 induces the pro-apoptotic bax gene (39, 40), whose product heterodimerizes with the apoptosis-antagonizing bcl-2 protein. Bax was shown to act as an effector of p53 in chemotherapy- and oncogene-induced apoptosis in fibroblasts, and contributed to p53-mediated tumor suppression in a brain tumor model (41–43). However, additional p53 target genes cooperating with bax were required for induction of a full apoptotic response by p53 in all systems described. Furthermore, studies of bax null mice demonstrated that bax is dispensable for induction of p53-dependent apoptosis in some cell types, e.g. T lymphocytes (44, 45).

p53 upregulates at least two cell surface death receptors from the tumor necrosis factor receptor (TNFR) family (46). Ligand binding to such death receptors triggers a cascade of signalling events resulting in activation of caspases and apoptosis. A p53-responsive element was identified within the first intron of the Fas/CD95/Apo1 gene, along with three putative elements within the promoter (47). Anticancer drugs induce Fas receptor expression in a p53-dependent manner in tumor cells of different origin, for example gastric, colon, and breast cancer, and hepatoma cell lines (47). Like bax, Fas is induced by p53 in a cell type- and signal-dependent manner (48, 49). p53 also activates another member of the TNFR family, KILLER/DR5, in signal-dependent manner (50). Its role in p53-mediated apoptosis and tumor suppression awaits elucidation.

Transactivation of the insulin-like growth factor binding protein 3 (IGF-BP3) gene is yet another mechanism by which p53 may promote apoptosis (51). IGF-BP3 is a secreted protein that binds insulin-like growth factor 1 (IGF1) and thereby inhibits IGF1-mediated mitogenic or survival signalling. Furthermore, Polyak *et al.* (52) have identified a series of new redox-related p53-induced genes called PIG's, whose products can induce formation of pro-apoptotic reactive oxygen species (ROS). Induction of PIG's cannot solely account for p53-dependent apoptosis, since ROS induction was observed in cells that did not undergo apoptosis, and expression of PIG3 alone failed to trigger apoptosis. Interestingly, transcriptional activation of the PIG3 gene depends on the presence of a Pro-rich region in p53 (53), previously shown to be important for p53-induced apoptosis (54).

Several other known p53 target genes are linked to apoptosis. Upregulation of the SH2/SH3 domain protein p85 by p53 may at least partially be responsible for oxidative stress-induced cell death (55). Although p85 is known to regulate PI3 kinase activity, it seems to use a PI3-kinase-independent pathway to transmit a death signal. Another pro-apoptotic activity of p53 is induction of cytoskeleton alterations. Disruption of microtubule structure is associated with induction of apoptosis, probably as a result of loss of anti-apoptotic activity of surviving and/or bcl-2 (56, 57). p53 activates the EF-1α gene shown to promote apoptosis, probably through its microtubule-severing function (58, 59). Generation of hypoxic conditions via prevention of angiogenesis is an indirect way of apoptosis induction by which p53 could delay tumor growth. p53 serves as anti-angiogenic factor through the transcriptional activation of thrombo-spondin I and brain-specific angiogenesis inhibitor GD-AiF/BAI1 genes (60–63). The PAG608/Wig-1 gene encodes a zinc finger protein that can induce apoptosis when overexpressed (64, 65), but its exact function remains unknown. p53 upregulates a number of other genes of unknown function (38).

Besides transactivation of target genes, p53 can repress transcription of some genes. The molecular mechanisms are poorly understood but do not appear to involve interaction of p53 with classical p53 DNA binding motifs. Genes downregulated by p53 include for example bcl-2, the IGF1 receptor, MAP-4, and presenilin 1 (66–69, 52). Downregulation of the anti-apoptotic bcl-2 protein should lower the apoptotic threshold, particularly in conjunction with the simultaneous p53-mediated induction of the pro-apoptotic bax protein. Transrepression of the IGF1 receptor gene is another mechanism for inhibition of IGF1 survival signalling in addition to transactivation of the IGF-BP3 gene (see above). Constitutive expression of the IGF1 receptor blocked p53-induced apoptosis, indicating that IGF1 receptor repression is important for p53-induced apoptosis, at least in some cells (67). Additionally, p53 downregulates the expression of a microtubule-associated protein, MAP4, which is able to stabilize polymerized microtubules (68, 70). Therefore, p53 may distort microtubule structure through both upregulation of EF-1α (see above) and downregulation of MAP4.

Finally, p53-induced apoptosis may involve transcription-independent mechanisms, as shown by the fact that p53-induced apoptosis occurred even in the presence of inhibitors of RNA or protein synthesis (71, 11). Possible transcription-independent pathways include p53-mediated increase of Fas trafficking from cytoplasmic stores to the cell surface (72). The recently described ability of p53 to induce caspase activation in cell-free extracts suggests that p53 can transduce apoptotic signals through direct protein-protein interactions (73, 74). Analysis of p53 deletion mutants have indicated two distinct domains that could be important for such interactions. Deletion of a Pro-rich domain located between the transactivation domain and the

core domain rendered p53 less efficient in triggering apoptosis (54, 75). The Pro-X-X-Pro motifs in this domain are present in a number of signal transduction molecules, serving as a binding site for SH3 domain-containing proteins. The binding of such molecules to p53 could play a role in p53-induced apoptosis. Deletion of the basic C-terminal domain was shown to impair p53:s apoptosis-inducing ability without affecting the transactivation function (76, 77). This domain was implicated in binding to DNA repair helicases XP-B and XP-D, suggesting that inhibition of DNA repair may be involved in execution of apoptosis by p53 (77).

From the above it is clear that p53 and the products of p53-regulated genes act at different levels, from extracellular and cell surface signalling down to the cytoskeleton, mitochondria, and the nucleus. However, all these processes do not necessarily occur within the same cell at the same time. It seems as if p53 achieves maximal efficiency in triggering of apoptosis through the recruitment of several molecular pathways in a cell type- and stress signal-dependent manner (Fig. 1). p53 target genes may be differentially activated or repressed depending on cell type and type of stress signalling (48, 78). The molecular basis for such differential promoter regulation could be for example modulation of the SST function of p53 by p53-interacting proteins expressed in a cell type-specific manner. In addition, p53 is differentially phosphorylated in response to different types of stress signals (79, 80). This could affect the ability of p53 to recognize specific p53 binding sites within p53-regulated promoters and thus have an impact on the transactivation of target genes (81, 82, 49). Therefore, p53 may trigger distinct apoptotic pathways depending on the type of stress signal.

TUMOR DEVELOPMENT AND THERAPY

A critical role of p53-induced apoptosis for p53-mediated tumor suppression *in vivo* has been demonstrated (6). Inactivation of p53-dependent apoptosis can presumably provide a selective advantage at any point during tumor progression. Oncogene activation and/or disruption of normal cell cycle regulation at early stages of tumor development induces p53 stabilization via ARF and subsequent p53-dependent apoptosis. In such cells, p53 inactivation should allow outgrowth of apoptosis-resistant variant clones and tumor progression. Hypoxia in rapidly growing solid tumors prior to the establishment of sufficient blood supply would also induce p53 and therefore favor p53-inactivating mutations and the emergence of more malignant clones. Similarly, treatment of clinically manifest tumors with DNA-damaging agents like irradiation or chemotherapeutic drugs should impose a selection for p53 inactivation and loss of p53-dependent apoptosis, the result of which may be resistance to therapy and tumor relapse.

p53 is inactivated by point mutation or deletion in around 50% of all human tumors. However, p53 function is likely to be deficient in many wild type p53-carrying tumors as well by a variety of mechanisms. Human papilloma virus (HPV)-carrying cervical carcinomas express the HPV E6 protein that binds p53 and promotes degradation of p53, thus eliminating p53-dependent apoptosis (83). Overexpression of the MDM2 protein that inhibits p53:s transcription transactivation function and promotes p53 degradation in cytoplasmic proteasomes blocks p53 function in some tumors, e.g. bone and soft tissue sarcomas (84, 85). Mutation of bax, an effector of p53-dependent apoptosis, has been found in certain colon carcinomas (86). In neuroblastomas, wild type p53 is sequestered in the cytoplasm and thus prevented from performing its normal function in the nucleus (87). ARF inactivation by homozygous deletion or point mutation occurs in human tumors with a frequency approaching that of p53 mutation (16), abolishing the p53-dependent apoptotic response to oncogenic signalling. p53 inactivation by one or another of these various mechanisms may account for loss of p53 function in a large fraction of the wild type p53-carrying tumors. Thus, it is possible that p53-induced apoptosis is disrupted in most, if not all, human tumors.

Yet the question remains as to why p53 is most frequently inactivated, rather than its downstream effectors. The reason is probably that p53 responds to a diversity of stress signals, and is the key trigger of a complex and multifactorial apoptotic response (Fig. 1). Therefore, one hit on p53 will inactivate several apoptotic pathways simultaneously. In contrast, inactivation of for example bax may only ablate one specific apoptotic pathway in some cell types. Likewise, ARF inactivation by homozygous deletion or point mutation would mainly affect p53-dependent apoptosis in response to oncogenic signalling and thus have less impact than loss p53 itself.

Since p53 is so often mutated in tumors, it is a prime target for therapeutic intervention. Reconstitution of tumor cells carrying mutant p53 with wild type p53 cDNA restores p53-dependent apoptosis. This approach has been applied clinically to patients with non-small cell lung cancer and head and neck squamous carcinomas with some success (for a review, see ref. (88)). Another approach, still at the experimental level, is reactivation of mutant p53 using short synthetic peptides (reviewed in ref. (89)). ARF acts synergistically with p53 to induce tumor cell apoptosis in response to DNA damaging drugs or ionizing radiation (27). This suggests that restoration of ARF function in tumors through gene therapy or drugs that mimick the effect of ARF on p53 could improve the efficacy of p53-activating therapy. Thus, studies of p53 and the molecular mechanisms behind p53-induced apoptosis may soon open avenues for more efficient anticancer

therapy. p53-induced apoptosis is still only partially understood, however, and future work will no doubt provide surprising new twists and clinically relevant insights.

ACKNOWLEDGMENTS

We thank Mikael Lindström for help with preparation of the manuscript. The authors are supported by grants from the Swedish Cancer Society (Cancerfonden), the King Gustaf V Jubilee Fund, and the Cancer Society of Stockholm.

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