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Apoptosis by p53: mechanisms, regulation, and clinical implications

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Biological properties of the p53 tumor suppressor

A loss of p53 tumor suppressor function is the most common event leading to the development of cancer. Genetic alterations in p53 have been identified in over 50% of different types of human cancer. A high incidence of cancer is observed in Li-Fraumeni patients who inherit a mutant allele of p53 [91]. Further, mice deficient for p53 (p53 null mice) develop various types of cancers at an early age [50]. These observations clearly demonstrate the importance of p53 as a tumor suppressor. The identification of p53 mutation can be used clinically for early diagnosis, prediction of prognosis and choosing the appropriate treatment for each cancer patient. Clinical interest in p53 led to an extensive study on the biochemistry and biology of its functions, with the aim of understanding how p53 suppresses the development and progression of cancer. This effort revealed the important role of p53 in converting external stress signals into appropriate biological responses through multiple functions. This review will focus on the function of p53 in mediating programmed cell death (apoptosis), with emphasis on the mechanistic and regulatory aspects, and their clinical implications. Other aspects of p53 have been recently reviewed [8, 27, 29, 32, 46, 50, 91].

The p53 protein exists in cells in a latent inactive form with a short half-life. However, upon exposure to stress signals, p53 undergoes a conformational change to its active form with a significant increase in its protein level. Stress signals that activate p53, include exposure to different types of damaged DNA, hypoxia, or a reduction in the ribonucleoside triphosphate pool [50]. Activation of p53 in most cell types induces cell growth arrest, primarily at the G1 phase, but also at the G2 phase [27]. In addition, p53 has been implicated in the spindle checkpoint which is important for maintaining ploidy [19]. Cell growth arrest induced by p53 is essential for allowing sufficient time for DNA repair prior to subsequent DNA replication. This response minimizes the accumulation of genetic errors during DNA replication or chromosome segregation, hence maintaining the integrity of the genome [50]. In cases of excessive DNA damage, the cell may undergo apoptosis thereby eliminating the accumulation of genetic alterations [7, 27].

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Biochemically, p53 is a transcription factor which can activate the expression of target genes containing p53 binding sites [46]. These genes mediate a variety of its biological activities including growth arrest (e.g., *p21*$^{waf\text{-}1/cip1}$), DNA repair (e.g., *GADD45*), apoptosis (e.g., *bax*) and anti-angiogenesis (e.g., thrombospondin). p53 can also repress the expression of certain genes, generally those containing a TATA box in their promoters, but lacking p53 binding sites. Examples include c-*myc*, *bcl-2* and proliferating cell nuclear antigen (PCNA) [30]. Transrepression by p53 is likely to result from binding to the TATA-binding protein (TBP) and consequently inhibiting its function as a basal transcription factor [46, 91].

Cell cycle arrest

The identification of p21 as a target of p53 provided new insight into the mechanism by which p53 induces growth arrest. Activation of p21 inhibits several cyclin-dependent kinases (CDKs) which are essential for driving the cell through G1 [82]. Specifically, inactivation of CDK4 and 6 prevents the phosphorylation of the retinoblastoma gene (pRb). In its hypophosphorylated form, pRb binds to and inhibits the function of the E2F transcription factor, a protein involved in the G1/S transition of the cell cycle. Hence, p53 induces cell growth arrest through a cascade involving p21, CDKs, pRb and E2F. Cells derived from p21-deficient mice can partially arrest in G1, suggesting that p21 is an important, but not the only, mediator of growth arrest by p53 [46]. The induction of other p53 target genes, such as *GADD45* may also contribute to growth arrest [46]. Overall, there is a good correlation between sequence-specific transactivation (SST) by p53 and the induction of cell growth arrest. However, it has been shown that p53 can also inhibit cell growth in the absence of SST [20, 33], indicating that this function of p53 may be more complicated, involving multiple mechanisms.

p53-mediated apoptosis and tumor suppression

The first demonstration of the apoptotic activity of p53 in myeloid leukemia M1 cells by Oren and colleagues [101] dramatically changed the view on tumor suppression by p53. Since 1991 extensive effort has been made to define the biological relevance of p53-mediated apoptosis in tumor suppression, and to understand the mechanisms involved. The apoptotic activity of p53 has been demonstrated in multiple cell types using a variety of experimental model systems and physiological conditions [7, 27]. Thymocytes from p53 null mice are resistant to irradiation, but not to other apoptotic stimuli such as glucocorticoids [98]. Thus, death induced by p53 is regulated and specific.

The link between the apoptotic function of p53 and tumor suppression has been established [27]. The most compelling evidence came from in vivo studies using genetically engineered mice. Symonds et al. [88] constructed transgenic mice bearing an SV40 large T antigen (LT) mutant that inhibits the function of the pRb family but does not affect p53 function. These mice developed choroid plexus tumors, but at a slow rate due to continuous p53-dependent apoptosis. Expression of the same transgenic LT mutant in p53 null mice resulted in aggressive tumor development, further demonstrating that tumor suppression is primarily due to p53-mediated apoptosis [7].

Mechanisms of p53-mediated apoptosis

The mechanisms by which p53 induces apoptosis are poorly understood, despite the formidable effort devoted to this subject over the past few years. While the role of SST in the induction of G1 arrest has been well established, its contribution to apoptosis has been a matter of controversy [7, 35]. In general, a correlation exists between the induction of apoptosis and transcriptional activation by p53. In fact, at least in some experimental systems, SST has been shown to be essential for the induction of apoptosis by p53 [5, 76, 102]. However, under certain cellular contexts SST was shown to be dispensable for p53-mediated apoptosis [7, 35]. The following sections overview the relevant evidence supporting the notion that p53 induces apoptosis by multiple mechanisms, which may co-operate.

SST-independent apoptotic function of p53

Several studies in the past few years assessed the direct contribution of SST to p53-mediated apoptosis. Initially, it was demonstrated that de novo RNA and protein synthesis are not required for p53 to induce apoptosis, at least in certain cellular systems [50]. More direct evidence came from analyzing the apoptotic potential of SST-deficient mutants of p53. A truncated form of p53, p53dl214, containing the first 214 amino acids of p53, induced efficient apoptosis in HeLa and Saos-2 cells in the absence of any measurable SST activity [35, 37]. These studies demonstrated that p53 can induce apoptosis without the induction of target genes; and this activity was found to be cell-type specific [38]. Recent findings provided further insight into the functional domain that may mediate the SST-independent apoptotic activity of p53. The N terminus of p53 contains five highly conserved proline motifs, a deletion of which impaired the ability of p53 to suppress cell growth, while retaining a full SST capacity [94]. Intriguingly, a spontaneous point mutation in one of these conserved proline residues was identified in a Li-Fraumeni family [87]. It remains to be elucidated whether this domain mediates the SST-independent apoptotic activity of p53.

The mechanism by which p53 induces apoptosis in an SST-independent manner is still not understood. All the experimental systems in which p53 was shown to induce apoptosis in the absence of SST share in common the loss of the pRb family function [35]. A direct consequence of this effect is the release of E2F [3]. The untimely activation of E2F-1, either alone or in conjunction with activated p53, may induce apoptosis in a p53-dependent manner [3]. The same may hold for deregulated c-*myc*, a proliferation promoting gene which induces apoptosis under growth restricted conditions [27, 98]. The direct contribution of c-Myc and E2F-1 to SST-independent apoptosis by p53 needs further demonstration.

Transcriptional repression by p53 has been implicated in the induction of apoptosis. This is based on the observation that several inhibitors of p53-dependent apoptosis, such as Bcl-2, E1B 19kD and the Wilm's tumor suppressor (WT1), relieve transrepression by p53 without affecting its SST activity or its ability to induce growth arrest [55, 81]. However, the correlation between transrepression and apoptosis does not hold in all cases [28]. Since p53 represses the expression of many genes [30], the contribution of specific gene repression to the induction of apoptosis is difficult to assess. Among the genes shown to be repressed by p53, *bcl-2*, insulin-like growth factor I receptor (IGF-IR) and MAP4, are the most relevant since they can overcome or attenu-

ate apoptosis induced by p53 [58, 61, 69]. Whether the repression of these genes directly contributes to the apoptotic function of p53 remains to be seen. Further, it would be interesting to test whether the SST-deficient mutants of p53 which induce apoptosis also retain the ability to transrepress these relevant genes.

The most likely mechanism for SST-independent apoptosis is through binding to other proteins which may mediate the apoptotic response. It is well established that p53 interacts with a variety of viral and cellular proteins [50], some of which may either modulate or mediate the activity of p53. For instance, p53 interacts in vivo with TFIIH, a basal transcription/DNA repair complex, and inhibits the activity of two helicases of this complex, XPB and XPD [96]. Intriguingly, the apoptotic activity of p53 is blocked in cells lacking XPB and XPD, in spite of retaining functional SST activity of p53 [96]. This suggests a role for XPB and XPD in the SST-independent apoptotic activity of p53. Several proteins have been identified by virtue of their ability to bind the N terminus of p53. One of these proteins, p53BP2, can also interact with the Bcl-2 protein. Interestingly, p53 and Bcl-2 compete for p53BP2 [63]; the outcome of this competition may influence the apoptotic response. The interaction of p53 with other synergizing proteins such as c-Abl and p300/CBP may also contribute to the apoptotic response (see section 'Regulation by viral and cellular proteins'). Overall, through binding to other proteins, p53 may mediate an apoptotic signal in the absence of SST. It should be noted that the alternative mechanisms outlined above for p53-mediated apoptosis are not mutually exclusive, but rather may act in concert.

SST-dependent apoptosis by p53

The studies described so far demonstrated the ability of p53 to induce apoptosis in the absence of transcriptional activation, but did not rule out the contribution of p53 target genes to this function. Indeed, kinetic studies revealed that apoptosis induced by SST-deficient mutants of p53 is slower and less efficient than apoptosis induced by wild-type (wt) p53 [37]. This suggests that the latter involves additional activity(s) of p53, most likely the induction of apoptotic target genes. A number of p53 target genes have been implicated in apoptosis. Among these *bax* plays a key role. Activation of Bax induces apoptosis by antagonizing Bcl-2 [48]. This induction of apoptosis suppresses tumor formation [100]. Indeed mutations in *bax* have been identified in colon cancers [70]. It has been estimated that Bax contributes approximately 50% of the total apoptotic effect of p53 in choroid plexus tumors [100]. However, thymocytes from *bax*-deficient mice undergo radiation-induced apoptosis in a p53-dependent manner, and overexpression of Bax did not restore radiation-induced apoptosis in the absence of p53 [11]. These observations indicate that the contribution of Bax to the apoptotic activity of p53 may depend on the apoptotic signals and the cell type.

Other relevant apoptotic targets of p53 include the insulin-like growth factor binding protein 3 (IGF-BP3) [46]. IGF-BP3 may contribute to p53-mediated apoptosis by antagonizing the survival effect conferred by IGF-1. This effect may be enhanced by the simultaneous repression of the IGF-IR by wt p53 [69]. In several cell types, p53 induces the expression of Fas/Apo-1 [66], a well-known mediator of apoptosis. Recent findings provide a functional link between Fas/Apo-1 and p53. Fas/Apo-1 is essential for drug-induced apoptosis of T cells, a process known to involve p53 [25]. Furthermore, activation of interleukin (IL)-1β-converting enzyme (ICE)-like proteases, CPP32 and Mch3a, has been detected during p53-induced apoptosis [14]. Whether this

activation is mediated through Fas/Apo-1 or by other pathways requires further clarification. Other p53 target genes implicated in the induction of apoptosis include *PAG608*, *E124* and *TGFa* [32, 44]. Their contribution to the apoptotic effect of p53 needs to be determined.

Taken together, it appears that the induction of p53 target genes plays a role in p53-mediated apoptosis. It is crucial to note, however, that only a subset of p53 target genes contribute to apoptosis, while other target genes may have the opposite effect. For instance, the presence of p21 is dispensable for the apoptotic ability of p53, but its induction can protect cells from death, presumably through the induction of viable growth arrest [26]. Therefore, the induction of apoptosis or growth arrest may require the activation of distinct sets of target genes. This notion gained support by p53 mutants having altered specificity for target genes. A mutant p53 derived from cervical carcinoma, p53Pro175, retains SST activity and can promote growth arrest, but is severely impaired in the induction of apoptosis. This biological phenotype correlates with the ability of p53Pro175 to induce the expression of p21 but not the apoptotic targets, Bax and IGF-BP3 [54, 74]. Similarly, p53Leu181 and p53Ala143 have an altered SST specificity, which explains their selective biological activities [24, 54]. The pathways leading to cell growth arrest and apoptosis seem, therefore, to be distinct.

A choice between viable growth arrest and apoptosis

The cellular decision to undergo either growth arrest or apoptosis in response to p53 is influenced by the complex interplay of intra- and extracellular factors [7]. Viral proteins that promote cellular proliferation, such as adenoviral E1A and human papilloma virus (HPV) E7, appear to trigger p53-mediated apoptosis [27, 34]. Both E1A and E7 inactivate pRb family members, thereby blocking growth arrest by p53. Moreover, reintroduction of pRb can block p53-dependent apoptosis [36]. This suggests that cells which receive growth inhibitory signals, but are unable to arrest properly, would opt to p53-dependent apoptosis. The inhibitory effect of other proteins, such as E1B 19kD, Bcl-2, and WT-1 may be directed specifically against the apoptotic activity of p53, without compromising its ability to induce G1 growth arrest [55, 81].

The cellular environment, in particular the presence of survival and growth factors, influences the susceptibility of cells to p53-mediated apoptosis. Activation of p53 in DA-1 and Baf-3 lymphoid cells by irradiation induces apoptosis in the absence of IL-3, but growth arrest in its presence [27]. Similarly, M1 myeloid leukemia cells and DP16 Friend erythroleukemia cells can be protected from p53-dependent apoptosis by IL-6 or erythropoietin, respectively [27]. The protection conferred by the survival factors may be associated with the ability of cells to enter growth arrest, although arrest at G1 per se does not guarantee survival [75].

A correlation exists between the intensity of the stress signals and the cellular response to p53. For example, upon exposure of DA-1 lymphoid cells to 11 Gy, the levels of p53 increased and were even higher after exposure to 33 Gy. In the presence of IL-3, exposure of DA-1 cells to 11 Gy induced viable growth arrest, while at 33 Gy the cells underwent apoptosis. Both responses are p53-dependent [27], suggesting that the level of p53 expression affects the cellular response. Indeed, Chen et al. [17] demonstrated that low levels of p53 induced growth arrest, whereas high levels promoted efficient apoptosis in an experimental system in which the level of p53 expression was controlled by a tetracycline inducible promoter.

In summary, the cell decision to undergo growth arrest or apoptosis depends on the cellular context, the cellular environment, the intensity of the stress signals, and the expression level of p53. The combination of these factors ensures that only cells that have the potential to recover from DNA damage will undergo DNA repair and survive. Cells which accumulate genetic changes, or are growing under stress conditions in an unsuitable physiological environment will be eliminated efficiently by apoptosis. This response minimizes the chance of accumulating additional genetic alterations and prevents the appearance of transformed cells.

Loss of p53 apoptotic function

The apoptotic activity of p53 is regulated by a variety of mechanisms. The most common and efficient mechanism for inactivating p53 function is through point mutations in the gene (see section 'Mutations in p53'). p53 activities can be regulated by post-translational modifications and allosteric modulation of its DNA binding ability [27, 86]. Cytoplasmic sequestration of p53 can also abrogate its growth inhibitory function [45]. p53 function may be compromised indirectly by proteins acting downstream to p53. Bcl-2 and E1B 19kD proteins block p53-mediated apoptosis by antagonizing Bax [98]. This section focuses on the direct modulation of p53 apoptotic function by viral and cellular proteins as well as by mutations.

Regulation by viral and cellular proteins

The SV40 LT and the E1B 55kD proteins block the apoptotic activity of p53 through protein-protein interactions [89]. The WT1 tumor suppressor protein also binds to and inhibits p53-dependent apoptosis in some cell types [55], but not in others [57]. A different mechanism is used by the HPV E6 protein which targets p53 for rapid degradation through the ubiquitin-proteasome pathway [27]. A growing number of other viral proteins that affect p53 function, have been identified. These include the adenoviral E4orf6, the hepatitis B virus HBxAg protein, and Epstein-Barr virus EBNA-5 and BZLF1 proteins [89]. Presumably, common to these viral proteins is their ability to overcome p53-mediated apoptosis, although through different mechanisms.

A key regulator of p53 is *mdm2*, an oncogene found to be amplified in sarcomas [34, 83]. Mdm2 binds p53 at the transactivation domain and blocks its SST activity. This interaction is obligatory for the regulation of p53 by Mdm2 [16, 38, 68]. Interestingly, the *mdm2* gene itself is transcriptionally activated by p53. Accordingly, through an autoregulatory feedback loop Mdm2 shuts off its own expression [68]. The time interval between the activation of p53 and the consequent induction of *mdm2* may define the window during which p53 exerts its growth inhibition [67]. Mdm2 can efficiently block p53-mediated apoptosis in certain cell types, but only partially in others [16, 38]. It appears that the extent by which Mdm2 inhibits p53-dependent apoptosis is largely dictated by the mechanisms through which p53 induces apoptosis in a given cell type [35, 38].

An additional and novel mechanism by which Mdm2 regulates p53 has been described recently. Mdm2 was found to promote the rapid degradation of p53 [39, 49]. This effect of Mdm2 requires interaction between the two proteins, and it appears to involve the ubiquitin pathway, since it can be overcome by proteasome inhibitors.

These findings provide the first demonstration of a cellular protein that regulates p53 stability in response to stress signals. Therefore, by modulating p53 at multiple levels, Mdm2 inhibits the effects of p53 and at the same time ensures that the signal generated by p53 is terminated. This tight regulation explains why tumors bearing an amplified Mdm2 tend to retain wt p53 [9, 18], and the dramatic lethality of *mdm2* null mice in the presence of wt p53 [59]. This information may be valuable in designing new strategies for cancer therapy (see section 'Restoration of p53 by blocking its interaction with inhibitory proteins').

The regulation of p53 described above results in the abrogation of its functions. However, the presence of synergizing proteins is required for normal p53 function, and can be further enhanced by specific regulators. For instance, p53 has been documented to interact with c-Abl [103]; an interaction which is strengthened by DNA damage and contributes to the growth inhibitory function of these proteins [97, 103]. c-Abl has recently been shown to induce apoptosis in response to DNA damage [104]. It is plausible that c-Abl and p53 may also synergize in the induction of apoptosis. Whether this synergism occurs, and contributes to the apoptotic activity of p53 needs to be demonstrated. An additional link between p53 and c-Abl may be mediated through another potential regulator of p53, ATM, a gene mutated in ataxia-telangiectasia [60]. Cells lacking ATM function are radioresistant and defective in accumulation of p53 and its ability to induce checkpoint arrest [60]. ATM binds to and activates c-Abl in response to radiation [79]. It is possible that ATM modulates p53 activity through activation of c-Abl, although direct regulation of p53 by ATM, for example by direct phosphorylation, has not been ruled out. Recently, it has been found that an interaction between the co-activators p300/CBP and p53 is essential for p53 function. The binding of p300/CBP to p53 enhances its SST activity, and contributes to p53-mediated growth arrest and apoptosis [6]. It would be interesting to evaluate the contribution of p300/CBP to the SST-independent apoptosis by p53.

Mutations in p53

Among different tumor types p53 mutation frequencies vary from high in lung, colon (over 50%), esophageal, ovarian and pancreatic cancer (40–45%), to moderate in renal and breast (20–30%), and to low in melanomas (10%). p53 mutations were rarely found in Wilm's tumors, testicular and pituitary cancers [91]. The vast majority of p53 mutations are missense single-base substitutions, leading to a full-length mutated protein with altered function [13, 34, 50, 83]. These mutations are commonly clustered in conserved regions (hot spots) within the central core DNA binding domain, and target amino acid residues that directly contact DNA or affect p53/DNA interaction, thereby abrogating specific DNA binding and SST by p53 [4]. The second most prevalent class of mutations affects the hydrophobic core of p53, resulting in structural alteration [83]. The type of mutation may be relatively unique for different cancer types and certain mutations are associated with specific carcinogens [34, 41, 83].

Mutant p53 proteins differ from wild type and from each other in conformation, localization and transforming potential. Some missense mutants, for example those mutated in codons p53His175, p53Trp248, p53Ser249 and p53His273, not only have lost the tumor-suppressive function of wt p53, but have also acquired additional activities, such as enhanced growth rate or resistance to chemotherapeutic drugs (gain of function [1, 51]). Since active p53 is a homotetramer, some p53 mutants, such as p53Val135

and p53His175, may prevent the activity of wt p53 by forming an inactive heterotetramer (dominant-negative effect [4]). This is in discrepancy with the classical two-hit model for tumor-suppressor genes in which both parental copies have to be inactivated. The high vulnerability of p53 as a target for genetic lesions in cancer rests partly on the large number of potential sites where minute damage can cause a major effect, and partly because mutations in one allele can compromise tumor suppression by wt p53.

Implications for diagnosis and methods of detection

There is still an emerging need for improved screening methods for early detection of human malignancies. Detection of p53 mutations may be useful in early diagnosis. In tumor types, such as head and neck, lung and breast tumors, mutations in p53 occur during the early stages of tumorigenesis, whereas in others, including gastrointestine, prostate and ovarian cancers, p53 mutations are found later in tumor development [91]. Knowing the status of p53 in tumor cells is important for predicting prognosis, and for choosing the proper therapeutic approach. Moreover, it can be used as a marker for tumor spread and for detection of rare residual tumor cells [83]. Accordingly, much effort has been devoted to developing techniques for rapid and efficient detection of p53 mutations.

The most precise method for detecting p53 mutations is by direct sequencing. The combined PCR/DNA sequencing technique can be used successfully for detecting p53 mutations in the urine of patients with preclinical bladder cancer, in the sputum of patients with early lung cancer, and in the detection of germ-line mutations in family members of Li-Fraumeni patients [83, 91]. Over 80% of all p53 mutations are found between exons 5 to 8, but sequence analysis of exons 2 to 11 is required for the detection of all the mutations [13]. An alternative technique is single-strand conformation polymorphism (SSCP), which is based on altered electrophoretic mobility of DNA sequences containing point mutations [91]. This method was shown to have a selectivity and specificity of ~90% for detecting p53 mutations [91]. However, negative results cannot be used to exclude p53 mutations and positive ones should be confirmed by DNA sequencing.

A different approach for detecting mutant p53 is based on the longer half-life of mutant p53 relative to the wt form [85]. Immunohistochemical staining (IHC) can detect stable p53 mutants but not wt p53. IHC is particularly useful for detecting mutations within the hot spots, but does not detect other types of mutations, such as deletions, insertions, and truncations [13]. Since positive results for p53 by IHC staining may reflect normal response of wt p53 to stress, or stabilization of wt p53 by viral and cellular proteins, the results must be verified by other techniques.

The presence of anti-p53 antibodies in a proportion of cancer patients (5–40%) is used for preclinical detection [85]. The advantages of this serological screening approach are the simplicity of the enzyme-linked immunosorbent assay, with no need for sampling tumor tissue, and that the level of p53 antibodies can be used to monitor the efficacy of therapy, as antibody titer tends to drop following treatments [85].

Finally, two functional assays have been described whose aim is to determine whether p53 is functionally active [22, 53]. The first is based on the induction of apoptosis following γ-irradiation (4 Gy) of peripheral blood lymphocytes, where patients with a germ-line defect in p53 have reduced apoptotic response [53]. The second is the

functional assay for the separation of alleles in yeast (FASAY), which measures the ability of p53 to induce the expression of a yeast ADE2 gene driven by a p53-responsive element. When grown on limiting adenine plates, wt p53 induces large, white yeast colonies, while mutated p53 leads to small, red colonies. This approach allows large scale screening even of samples mixed with normal tissue [22].

Implication for prognosis and therapy

p53 status and prognosis

Knowing the status of p53 in cancers should be helpful for predicting prognosis and for choosing the best therapeutic regimen. This is based on the findings that p53 mutations alter the cell's ability to undergo growth arrest and apoptosis, and its response to radio- or chemotherapy; some mutations may even enhance tumorigenicity. In general, elevated p53 expression has been associated with a poorer prognosis for tumor types, including breast, colon, gastric, and non-small cell lung cancer [83, 91]. p53 alterations have been implicated in the progression from chronic phase to blast crisis in chronic myelocyte leukemia, or from Barrett epithelium to invasive esophageal cancer, and also in the poor response of bladder cancer to BCG (Bacillus of Calmette and Guérin), and increased vascularization of lung tumors [12, 23, 83, 91]. By contrast, no correlation was observed between p53 status and survival of patients with soft tissue sarcoma [62], or the risk of breast cancer relapse following surgery and radiotherapy [84]. These discrepancies may be explained, at least in part, by the observation that mutations within the zinc-binding domains (L2 and L3) are associated with poorer prognosis in breast cancer, while mutations outside these domains have no prognostic value [1].

It is generally accepted that tumor cells bearing mutant p53 are more resistant to chemotherapy than those expressing wt p53 [1, 8, 80, 91]. However, some evidence suggests that p53 status may not necessarily predict the therapeutic outcome and, moreover, some tumor cells with mutant p53 may be particularly sensitive to certain DNA damaging agents [93]. By itself, the mere knowledge of p53 status may be of limited value with regards to predicting prognosis and designing therapeutic treatments. For this purpose, the determination of the "apoptotic competence" of the tumor cells may be more helpful [80].

Therapeutic approaches on the basis of p53 status

The efficacy of anti-cancer drugs correlates with the ability of cells to undergo p53-dependent growth arrest or apoptosis. Tumor cells bearing non-functional p53 often fail to respond to chemotherapy. Accordingly, an important strategy for tumor therapy is to reconstitute the apoptotic function of p53 in these cells. Several studies have shown that restoration of wt p53 in tumor cells that contain mutant or no p53, induced either apoptosis or growth arrest, but had no effect on normal cells or tumor cells with functional p53 [77]. These findings support the concept of p53 gene therapy. For this approach to be clinically applicable, targeting p53 to the tumor cells must be efficient and the level of p53 expression should be sufficiently high to overcome the dominant negative effect of the endogenous mutant.

Reconstitution of wt p53 by viral and non-viral vehicles

The most common delivery vehicles for gene therapy have been viral vectors [72], although non-viral vehicles such as transferrin-modified liposomes have also been used [99]. For the treatment of human cancer cells in mice, p53 was delivered in replicative-defective retroviruses or adenovirus serotype 5 [72]. Successful growth suppression has been achieved by treatment of various tumor types, such as breast, cervical and lung cancer [72]. Growth inhibition by p53 was shown to be enhanced by the simultaneous transfer of another growth inhibitory gene, $p16^{INK4}$, an inhibitor of CDK4/6, or in combination with chemotherapy [64, 77]. Delivering genes into cells can be simplified by chemically linking the expression plasmid for p53 with the capsid of the adenovirus [65]. This adenovirus/DNA complex provides a general delivery vector that is simple to construct and which can be used to test the effect of a single or a combination of multiple therapeutic genes on malignant cells.

Due to their success in animal models, retroviral and adenoviral vehicles expressing wt p53 have been approved for phase I protocol in humans [34, 72]. Intratumoral p53 delivery by adenovirus serotype 5 has been approved for head and neck squamous carcinoma, for non-small cell lung cancer in combination with chemotherapy, and as hepatic artery infusion for primary and metastatic liver tumors. Further, p53-DNA injection in colorectal liver metastasis and hepatocellular carcinoma has been used in humans [72]. A phase I study of retrovirus-mediated wt p53 gene therapy of lung cancer in human had only a partial and local effect [73]. So far, these trials have been limited to patients with advanced incurable cancer, therefore giving less encouraging results. Regardless of this limitation, the viral therapy approach still suffers major disadvantages. First, not all cells can be transduced by adenovirus, hence the approach may be limited to a subset of malignancies. This may potentially be overcome by substituting endogenous viral surface glycoprotein with a tissue targeting molecule [72]. Second, adenoviruses are immunogenic, thus, limiting repeated administration [72]. Insertion of the adenoviral E3 into a recombinant vector, decreased immunogenicity and increased duration of gene delivery [43]. Third, this approach has a low penetration into deeper tumor layers. Overall, a higher clinical response should be expected in the treatment of residual tumor.

Restoration of p53 by blocking its interaction with inhibitory proteins

Restoration of wt p53 might be less efficient in tumors bearing viral oncogenes (e.g., E6 in cervical cancers) or endogenous cellular proteins (e.g., Mdm2 in sarcomas). Since E6 and Mdm2 promote p53 degradation [27, 39, 49], blocking their interaction with p53 should elevate the level of functional p53. Several approaches have been used to overcome the inhibitory effect of these proteins. First, the level of the inhibitory protein can be reduced by antisense therapy. Significant reduction in tumorigenicity was achieved in mice by adenovirus-mediated transfer of antisense RNA to E6 and E7 [31]. Similarly, antisense to *mdm2* reduced Mdm2 protein levels in a human glioblastoma with subsequent increase in cisplatin susceptibility [47]. Second, the interaction can be blocked by antibodies directed to the interaction site or peptides encompassing this site. Böttger et al. [10] found peptides that efficiently disrupt p53-Mdm2 interaction, thereby, releasing active p53 molecules. A similar effect was achieved by microinjection of an antibody to the p53-binding domain of Mdm2 [9].

Third, the interaction with the inhibitory protein can be avoided using p53 mutants that fail to bind the inhibitory proteins but retain growth inhibitory function [52].

Activation of mutant p53 by peptide treatment

It is well accepted that the specific DNA binding capacity of wt p53 is negatively regulated by its C-terminal domain by an allosteric mechanism, thus keeping p53 in a latent inactive form. p53 can be converted from a latent to an active form by several mechanisms including specific phosphorylation of serine residues in the C terminus [86], truncation of the C terminus, binding of PAb421 antibody to its C-terminal epitope, or with a peptide harboring both the phosphorylation site and the PAb421 epitope (C369-383 [42]). Thus, the relief of the C terminus suppression activity, by covalent or non-covalent modifications, is an important step in p53 activation. Intriguingly, subsequent studies showed that not only wt p53, but also a subset of p53 mutants, can be activated by PAb421 [2]. These observations lend support for the idea of reactivating mutant p53 in tumor cells, and thereby, restoring the growth inhibitory activity by p53. Recently, Selivanova et al. [78] were able to restore SST, growth inhibition and apoptosis in tumor cells bearing mutant p53 by introducing a peptide containing residues 361–382. The peptide effect was specific to mutant p53 since it was inert in p53-null tumor cells [78]. This approach is likely to be most successful for conformational mutations within the sequence-specific DNA binding domain.

Specific killing of p53-defective tumor cells by mutant adenovirus

A novel approach of treating cancers bearing non-functional p53 is based on a mutant adenovirus that lacks the viral E1B 55kD protein (ONYX-015) [40]. The E1B 55kD protein inhibits p53 activity and consequently prevents E1A-mediated apoptosis. The ability of wt p53 to limit virus replication provides the rationale for the tumor-specific effect of ONYX-015. Because ONYX-015 cannot effectively counter p53 activity, it is unable to replicate in normal cells or tumor cells with functional p53. On the other hand, in tumor cells deficient for p53, E1B 55kD is not required for viral replication. Consequently, ONYX-015 selectively kills p53-aberrant tumor cells. The released virus can spread and continue the cycle of destruction provided that it infects other p53-deficient tumor cells. The potential for virus spread distinguishes this strategy from other gene therapy approaches, which are limited by the efficacy of gene transfer. ONYX-015 is effective, at least to some extent, in a variety of tumor types, including carcinomas of the breast, colon, ovary and cervix as well as certain drug-resistant tumor cells [40]. Further, no toxic effect was observed in normal cells. The major problem with ONYX-015 is its immunogenicity which limits virus spread. This may be overcome by a concomitant treatment with immunosuppressive drugs. Phase I clinical trials using ONYX-015 are underway.

p53 vaccines in the prevention of tumor progression

Vaccination against tumors bearing mutant p53 is based on the high expression of p53 that can be presented as peptide epitopes by tumor MHC in quantities sufficient to be

recognized by p53-specific cytotoxic T lymphocytes (CTLs). Immune responses to p53 may be induced by p53-derived peptides, p53 expressed in a recombinant vaccinia virus, or by immunization with DNA encoding for p53 protein sequences [15]. Immunization of HLA-A2.1 transgenic mice with p53-derived peptides elicited a CTL response specific to human tumor cells harboring mutant p53 in vitro. Increased resistance to tumor challenge was observed in mice immunized with p53-derived peptides, dendritic cells pulsed with a p53 peptide, recombinant canarypox virus expressing p53 (ALVAC), or constructs containing full or partial p53 sequences [15, 56, 71]. However, the anti-tumor response in animals bearing pre-existing tumors is poor. Since the immunization with wt or mutant p53 is equally effective [56, 71], there is no need for developing a tumor vaccine directed to each specific p53 mutant (over 700 known). Thus, overexpressed p53, although seen as self, can be used as a target for immunotherapy. It should be emphasized, however, that successful immunization in mice was achieved only with human-derived p53 peptides sharing little homology to the mouse sequence. Thus, a major problem with this approach is the possibility that tolerance to p53 might prevent proper CTL response to p53 in humans [90]. Clinical trials are underway in cancer patients using peptides bearing the specific p53 mutant sequence for each patient's tumor [15].

Treatment with drugs inducing p53-independent apoptosis

A major obstacle in tumor chemotherapy is the drug resistance of several types of tumor, mainly due to their inability to undergo p53-dependent apoptosis. One strategy would be to choose drugs that also promote apoptosis in a p53-independent manner, including etoposide [21], paclitaxel (Taxol) [93], vincristine and doxorubicin [34]. Recently, a novel drug, 7-hydroxystaurosporine (UCN-01), which inhibits protein kinase C and abrogates the G2 checkpoint, has entered phase I clinical trials [95]. UCN-01 markedly enhanced killing of tumor cells rendered p53-defective by γ-irradiated or cisplatin, and may therefore be effective in treating tumors lacking functional p53 [95].

Overall, several different approaches have been shown to be successful in cancer therapy, although to varying extents. Experience so far suggests that combined treatments are more effective. For example, combination treatment of ONYX-015 with chemotherapeutic agents (cisplatin, 5-fluorouracil) is more effective than either therapy alone [40]. In addition, the effectiveness of p53 gene therapy may be enhanced by combining it with a simultaneous inhibition of a negative regulator of p53-dependent apoptosis, such as Bcl-2 and Mdm2. Encouraging results were obtained in mice treated with oligonucleotide antisense to *bcl-2* [92].

Conclusion

The p53 molecule plays a pivotal role in the regulation of cell cycle and cell death. The activation of p53 function triggers cell growth arrest or apoptosis (Fig. 1). The final outcome depends upon the intensity of the stress signal, the extent of DNA damage, the level of p53 expression, the cellular context and the delicate balance between factors that promote or inhibit apoptosis in the intra- and extracellular environment. p53 induces apoptosis by multiple mechanisms, involving SST-dependent and -independent pathways. Various mechanisms have evolved by which p53 activity is abrogated

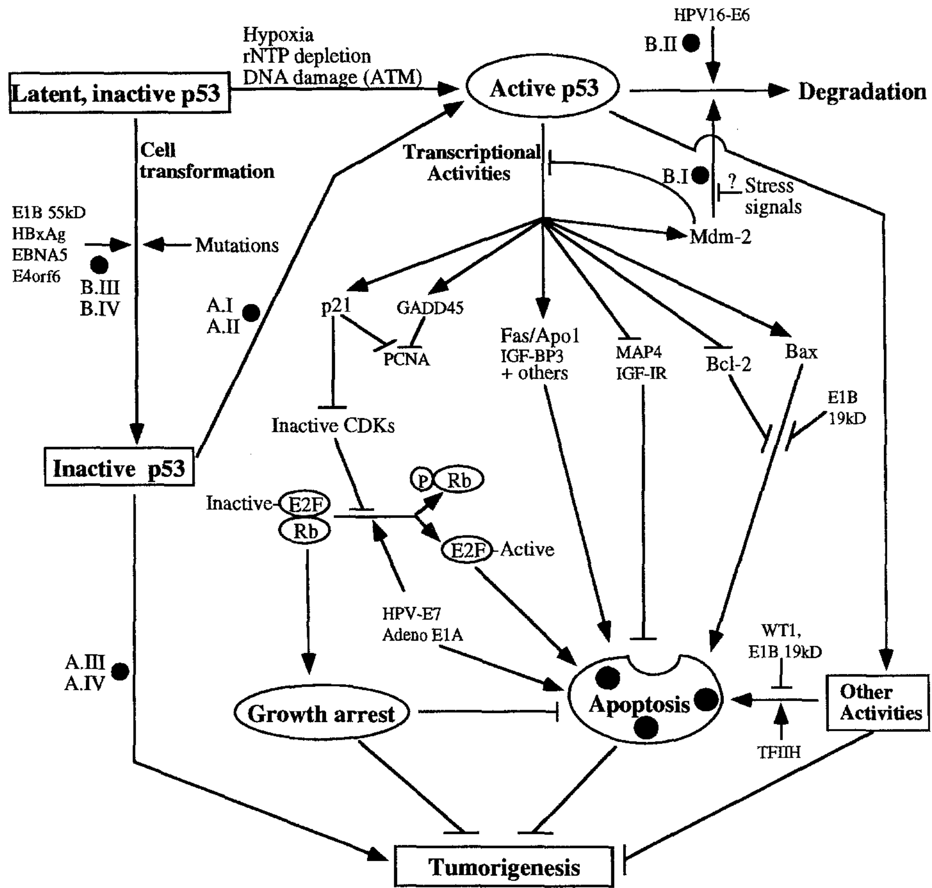

Fig. 1. Following stress, wild-type p53 is activated and induces either growth arrest or apoptotic cell death, thus suppressing carcinogenesis. However, tumor cells with non-functional p53 fails to undergo p53-dependent apoptosis and growth arrest. Several therapeutic strategies have been developed to reconstitute p53 function in tumor cells and to induce apoptosis in a p53-independent manner. Sites of possible therapeutic intervention are indicated by ● together with references to the appropriate paragraph in Table 1. *Lines* ending in an *arrowhead* indicate induction, whereas those ending in a *bar* indicate inhibition

by viral and cellular proteins. The most efficient mechanism for a loss of p53 function is through mutations in p53, frequently found in human cancers. Determining the status of p53 can, therefore, be used for early diagnosis, at least for some tumor types. Among the detection techniques, direct sequencing and the functional apoptotic assay are the most informative. Since a loss of p53 function tends to reduce sensitivity to radio- and chemotherapy, knowing the status of p53 may also help in designing correct therapeutic treatments. The choice of therapy depends on whether p53 is inactivated by structural missense mutation, by deletion/insertion mutations or by interaction with viral and endogenous cellular proteins (Table 1). Therefore, it is crucial to define additional genetic alterations in the same tumor. Novel strategies for cancer therapy aim to restore p53 activities by either introducing p53 exogenously or by activating endogenous p53 whether it is wt or mutant. Alternative strategies are targeted towards killing

Table 1. Strategies for tumor therapy based on p53 status

Approaches	Methods/drugs
A. Treatment of tumor cells bearing mutant p53	
I. Reconstitution of wild-type p53[a]	Adenoviral vector Retroviral vector Transferrin-liposome
Combined treatment with:	p16$^{INK4/CDKN2}$ Chemotherapy[a] Antisense to oncogenes, e.g., Bcl-2
II. Activation of mutant p53	Synthetic peptide to the C-terminus
III. Apoptosis specific to tumor cells with mutant p53	E1B 55kD-deficient adenovirus (ONYX-015)[a]
IV. Induction of p53-independent apoptosis	Doxorubicin[b], etoposide[b], paclitaxel (Taxol)[b] vincristine[b], 7-hydroxystaurosporine (UCN-01)[a]
V. Introduction of p53 target proteins	Delivery of Bax^{-}
B. Treatment of tumors with inactivated wild-type p53	
I. Disruption of p53/Mdm2 interaction (sacromas)	Synthetic peptides to the binding site Antisense to *mdm2*
II. Prevention of HPV16 E6 expression (cervical cancer)	Antisense to E6
III. Relief of viral-mediated inhibition of p53 function	Antisense to E1B 55kD, HBxAg or EBNA5.
IV. Reconstitution with functional variants of p53	Delivery of functional p53 refractory to inactivation
C. Tumor vaccines against cancers with accumulated p53 protein[a]	
I. Induction of cellular immune response by	p53-derived peptides Vaccinia vector expressing p53 Immunization with DNA encoding p53 sequences

[a] In clinical trials
[b] Clinically accepted drug

tumor cells lacking functional p53 (Table 1, Fig. 1). Some of these therapies have entered phase I trials (Table 1). A better outcome may be expected when combining these strategies with conventional chemo- and radiotherapy.

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