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New Insights into p53 Regulation and Gene Therapy for Cancer

Alain G. Zeimet, Karin Riha, Jennifer Berger, Martin Widschwendter, Martin Hermann, Günter Daxenbichler and Christian Marth*

Department of Obstetrics and Gynecology, University Hospital, Innsbruck, Austria

ABSTRACT. Due to its critical involvement in cell cycle control and apoptotic signaling, the transcription factor p53 has become the most important tumor suppressor currently under investigation. *TP53* is the most frequently mutated gene in human cancers and is thought to play a crucial role in malignant transformation. Therefore, p53 appears to be an appealing target for gene therapy. Adenoviral-based p53 gene transfection is now being introduced in large clinical trials. Viral cell entry was found to be the rate-limiting step of gene delivery and thus of therapeutic efficiency. Attachment of adenoviruses to the target cell surface is mediated through the coxsackie-adenovirus receptor, and internalization is achieved via interactions with integrins of the $\alpha v\beta_3$ and $\alpha v\beta_5$ class. The assumption that the restitution of the p53-dependent apoptotic pathway results in a higher responsiveness of solid tumors to cytostatic agents remains a major matter of debate. Combinations of p53-based gene therapy with other components involved in apoptosis, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/APO2L, or agents neutralizing tumor-promoting antiapoptotic signals, such as humanized anti-growth factor antibodies, should further improve the effectiveness of cancer treatment in the future. BIOCHEM PHARMACOL **60**;8:1153–1163, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. p53; gene therapy; ovarian cancer; chemosensitivity; adenovirus receptor

Malignant transformation is closely related to mutations, giving rise to oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function. *TP53*, the gene encoding the transcription factor p53, is the most frequently mutated gene in human cancers. The observations that p53 was found in high amounts in a variety of transformed cells as compared to its generally low expression in normal cells [1–3], together with the immortalizing ability of p53 cDNA when transfected into normal cells either alone or in cooperation with *ras* [4, 5], originally led to the assumption that p53 represents a dominantly acting oncogene. However, subsequent studies have revealed that elevated levels in transformed cells were not the result of gene overexpression, but rather were caused by the increased stabilization of normally short-lived p53 [6] and that the cloned p53 cDNAs used in the early experiments contained dominant negative missense mutations within regions important for the biological activity of p53 [7]. In contrast to the initial idea of p53 function, there is conclusive evidence to date that in normal cells wild-type p53 is critically involved in cell cycle control, DNA repair, and apoptosis, and that the loss of its normal function constitutes an important step in the malignant transformation process. Because of its physiological role in the maintenance of genetic stability, p53 has also been designated "the guardian of the genome" [8]. P53 has emerged as one of the most important molecules currently under investigation, and a plethora of *in vitro* and *in vivo* data provide insight into the complexity of the "p53 machinery." Moreover, functionally related but distinctly regulated pathways, such as mitogen- or stress-activated kinases (MAP, SAP), cyclin-dependent kinases (cdk), the Bcl-2 family, Rb† tumor suppressor, and the E2F/ARF system, can directly or indirectly influence the various steps of the p53-signaling cascade in an agonistic or antagonistic manner. Despite the complexity of these interactions, p53 is considered a central molecule in the control of critical cellular functions, and thus repairing aberrant p53 pathways in cancer cells holds exceptional promise for the development of new, safer, and more effective cancer treatments for the future.

p53-based gene therapeutical *in vitro* and *in vivo* approaches, which aimed at a direct correction of the specific molecular defect in p53-mutated malignant cells, showed notable efficacy in numerous cancer types, including head and neck, lung, prostate, cervical, and ovarian cancer [9–13]. The restitution of normal p53 function in malignant cells increases their sensitivity to undergo apoptosis,

* Corresponding author: Dr. Alain G. Zeimet, Dept. of Obstetrics and Gynecology, University Hospital Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria. Tel. +43-512-5043050; FAX +43-512-5043055; E-mail: alain.zeimet@uibk.ac.at

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† *Abbreviations:* Rb, retinoblastoma; CAR, coxsackie-adenovirus receptor; aa, aminoacids; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; and rAd-p53, recombinant p53 wild-type-carrying adenovirus serotype 5.

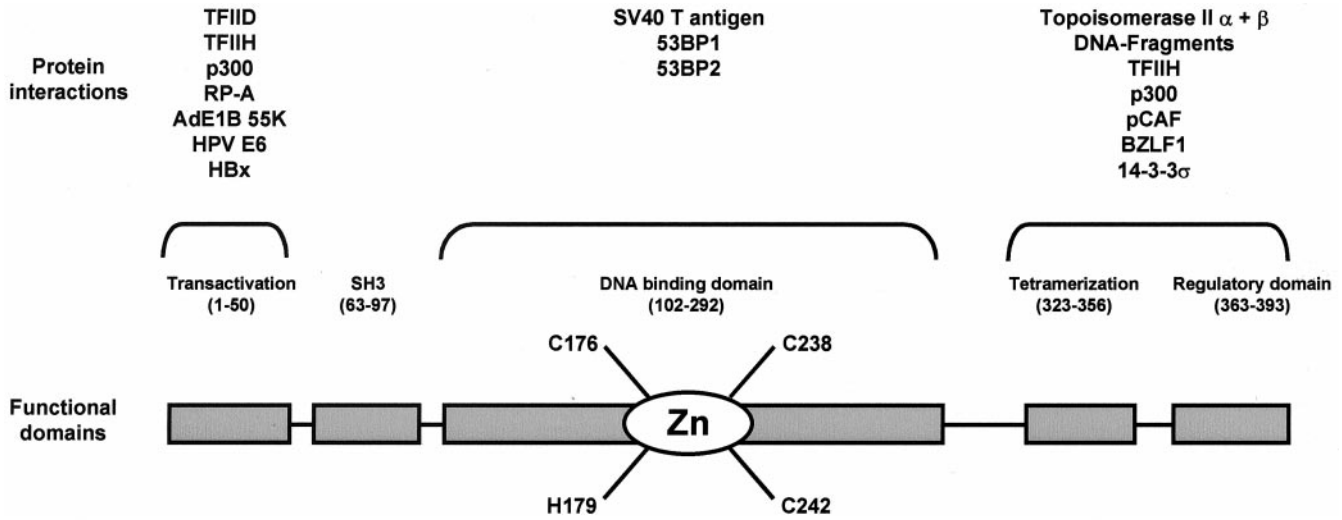

FIG. 1. Schematic illustration of the functional domains of the p53 protein. At the top, cellular and viral proteins known to interact with the various regions of p53 are indicated. The numbers in parentheses refer to the aminoacid residues. HPV E6, human papilloma virus early protein 6; HBx, hepatitis B virus X protein; SV40, simian virus 40; 53 BP, p53-binding protein.

which may be paralleled by an increased sensitivity to chemotherapeutic drugs, whose cytotoxic effects are presumed to be dependent on induction of apoptotic cell death. This hypothesis was taken into consideration in a number of protocols of clinical studies combining p53-based gene therapy with cytostatic agents.

In current studies, recombinant adenoviral vectors are mostly favored over alternatively available viral vector systems for gene delivery to tumor cells [9–13]. In several clinical gene therapy trials, however, relative resistance of target tissues to adenoviral infection has been noted [14]. Deficiency of the primary adenovirus receptor CAR and/or integrin dysregulation in tumor cells has been understood to be the biological basis of this phenomenon [15, 16]. New generations of genetically modified adenoviral vectors and non-viral vectors, such as liposomal systems, are considered very attractive for future *in vivo* approaches.

FUNCTIONAL ASPECTS OF p53 PROTEIN

Although under certain circumstances *trans*-regulatory-independent mechanisms may be of significance for p53 function, there is compelling evidence that *trans*-regulation of downstream genes is essential for p53 action, especially tumor suppression. After activation by a variety of stimuli, including cytotoxic agents, radiation, oxidative stress, hypoxia, nucleotide depletion, oncogene activation, but also physiological mitogenic stimulation, p53 binds in tetrameric form [17, 18] to four palindromic copies of its consensus sequence [19]. Recently, the Vogelstein group extended the p53 target gene family by 31 genes that were not previously known to be regulated by p53. This report also revealed a considerable heterogeneity in the transcriptional responses to p53, even in cells derived from the same stem cell type, and provided insights into the pleiotropic biologic effects of cell-specific responses to p53 [20]. The number of downstream genes *trans*-repressed and *trans*-activated by p53 is presently estimated at 70 and 80, respectively [21].

Human p53 protein consists of 393 aa and has the general molecular structure of a transcription factor with a transcriptional activation domain at the N-terminus (aa: 1–42), a sequence-specific DNA-binding domain within the central part (aa: 102–292), and an oligomerization domain (aa: 323–356) together with a regulatory domain (aa: 360–393) at the C-terminal portion [19–24] (Fig. 1).

Besides its function allowing p53 to recruit the basal transcriptional machinery, the transactivation domain also plays a key role in the rapid degradation of p53 through the ubiquitination pathway induced by the binding of a number of proteins, including viral proteins such as human papillomavirus E6 and adenoviral E1B-55-kDa protein, as well as the intrinsic regulator of intracellular p53 levels, namely the human mdm2 [25–28]. The *mdm2* gene itself, on the other hand, is *trans*-activated by p53, resulting in a negative feedback control of p53 activity [29]. Recently, however, high p53 levels were shown to *trans*-repress *mdm2* gene activity through promoter 3 located in intron 3 of this gene [30]. This finding adds a further element to the autoregulatory feedback loop between p53 and mdm2.

The DNA-binding domain is folded into three loop–helix structures directly involved in DNA contact [31, 32]. Although the loops are connected and stabilized by a divalent zinc atom, this structure differs substantially from the classical "zinc fingers" of other transcription factors and seems to be conserved exclusively in the recently identified p53 homologues p73 and p63 [33]. Notably, 80%–90% of the p53 mutations in malignant cells are located within this central part of the molecule.

The fact that tetramerization is required for adequate *trans*-regulation of downstream genes *in vivo* and for p53-mediated inhibition of carcinoma cell growth [18] under-

lines the importance of the oligomerization domain for the biological function of the molecule. The extreme C-terminal regulatory domain appears to negatively regulate p53 sequence-specific DNA binding [24]. This notion is supported by the finding that the C-terminal-recognizing polyclonal antibody PAb421 is able to activate specific DNA binding [34]. Recently, Kim *et al.* reported evidence that in addition to the regulatory domain at the extreme C-terminal, the N-terminal proline-rich aa sequence 80–93 may be involved in DNA-binding repression in a cooperative manner. Free synthetic peptide corresponding to aa sequence 361–382 of C-terminus, when experimentally inserted into cancer cells, is presumed to bind the proline-rich sequences 80–93, and this intercalation between both genuine repressor regions causes conformational changes in the p53 molecule, leading to rapid induction of apoptosis. Of special note is that this apoptosis is mediated via the Fas (APO1)/Fas ligand pathway and that this phenomenon was selectively observed in malignant cells overexpressing p53 or carrying mutant p53, but not in cells with wild-type or complete loss of p53 [35]. As shown in Fig. 1, the C-terminal domain can also physically interact with a number of viral and cellular proteins, including hepatitis B virus X protein, 14-3-3σ, and several helicases involved either in transcriptional or DNA repair processes [36–38]. Recently, human topoisomerase II has also been shown to bind to the regulatory sequences of the C-terminal region. This fact is suggested to play a substantial role in the induction of apoptosis by topoisomerase II inhibitors such as etoposide and doxorubicin, which cause accumulation of p53-interacting enzyme–DNA adducts [39].

Following DNA damage or other stress stimuli, p53 protein rapidly accumulates and becomes activated. As the increase in intranuclear p53 protein occurs as soon as 30 min after a DNA-damaging event, it must be postulated that posttranscriptional mechanisms, involving increased p53 mRNA translation, play a major role in the rapid up-regulation of p53. In this context, a translation repressor element residing in the 3′ untranslated region of the human p53 mRNA was recently identified together with a specific 40-kDa-binding protein acting as a *trans*-repressor [40]. Furthermore, it has been speculated that p53 itself can interact with its own mRNA to inhibit translation [41].

Disruption of the mdm2 degradation pathway, however, is considered the most important mechanism leading to rapid stabilization and accumulation of p53 [28]. Stress-induced serine phosphorylation of p53 at the N- and C-terminal regions is regarded as a key step in destabilizing the interaction of p53 with mdm2 [42]. In addition, there is evidence that E2F-controlled ARF protein represents a reliable inhibitor in mdm2-mediated p53 degradation. The mechanisms do not appear to be mutually exclusive, and it was suggested that phosphorylation may be essential in the initial induction of rapid p53 activation after genotoxic stress, followed by long-term stabilization of p53 mainly mediated through ARF expression [43]. However, recent observations revealed that significant mdm2 reduction, and not phosphorylation, plays the most important role in p53 activation in response to stress. This is consistent with the findings that mutations of all phosphorylation sites in p53 protein do not reduce the ability of genotoxic agents to induce p53 activation [21].

In addition to these fundamental mechanisms in p53 up-regulation, enhancement of *TP53* gene transcription, through cooperation by activator protein-1, nuclear factor-kappaB, and MYC/MAX, was recently proposed as a third pathway [44].

Regarding the primordial functions of p53, one crucial question remains: *What determines whether a damaged cell, after p53 activation, undergoes cell cycle arrest with subsequent DNA repair or apoptosis?* Although the exact molecular mechanisms governing this phenomenon have not been completely elucidated, it seems that the quality of stress or damage and its (non-)reversibility, together with the coinciding cell cycle phase and the cell type concerned, are some kind of determinant. This is in agreement with the observations that the kinetics and duration of p53 accumulation vary considerably in different cell types in response to various damaging agents. It is well established that p53-dependent cell cycle arrest in the G1 phase requires transcriptional activation of the cyclin-dependent kinase (cdk) inhibitor $p21^{WAF1/CIP1}$ [45]. Whether $p21^{WAF1}$ is an absolute prerequisite for G1 arrest remains a matter of debate, as $p21^{WAF1}$ (-/-) rodent fibroblasts are only partially deficient in G1 arrest [46], while in human p53 wild-type tumor cells, G1 arrest was completely abrogated in the absence of $p21^{WAF1}$ [47]. The best-characterized downstream target of the G1 inhibitory activity of $p21^{WAF1}$ is the repression of cyclin-dependent phosphorylation of the Rb protein, which in its hypophosphorylated state sequesters the transcription factors of the E2F family known to promote the S phase [48]. As Rb null mice fibroblasts remain partially functional in their G1 arrest [49], it is tempting to speculate that, besides Rb, additional targets mediate p53 G1 growth arrest. Nonetheless, the crucial role of the E2F family was underscored by the findings that overexpression of E2F-1 is able to overcome radiation-induced G1 arrest [50, 51]. Interestingly, the deletion of $p21^{WAF1}$, as well as high cellular E2F levels obtained by inactivation of Rb, can cause cells that would otherwise undergo G1 arrest to commit apoptosis in response to DNA damage [52]. p53 is also thought to be partially involved in the G2 checkpoint through 14-3-3σ *trans*-activation, but recent data have indicated that 14-3-3σ is mainly under the transcriptional control of the p53 homologue p73α [20].

The pathway leading to p53-dependent apoptosis is not as well understood, but is believed to involve both transcriptional as well as transcription-independent mechanisms. Bax, which is known to antagonize the antiapoptotic activity of Bcl-2, is the best-studied mediator of p53-dependent cell death [53, 54]. JMY, a recently discovered novel cofactor for p300, was shown to be involved in the activation of the *BAX* gene. It appears that the presence of certain alternatively spliced variants of JMY in the p300/

CBP (CREB-binding protein) transcriptional coactivator multiprotein complexes determines at least in part whether p53 mediates cell cycle arrest or apoptosis [55]. However, E2F1–DP1 complexes, which were recently shown to be regulated by mdm2, may through $p19^{ARF}$ induction lead to quantitative or qualitative changes in p53 and thus account for an indirect apoptotic signal. In addition, certain members of the E2F family at high relative levels are endowed with properties that directly induce apoptosis [56, 57], possibly by overriding the cycle arrest pathway [58]. Neutralizing the survival and mitogenic signals of insulin-like growth factor (IGF)-1 through a p53-induced up-regulation of its IGF-binding protein 3 is also involved in the apoptotic p53 pathway [59]. Members of the TNF receptor superfamily, especially Fas/APO1 and TRAIL/DR5, were also found to be involved in p53-dependent apoptosis, but not cell cycle arrest [35, 60, 61]. Besides the transcriptional up-regulation of both membranous receptors by p53 [20], there is some evidence, at least for Fas, that p53 is able to induce a rapid transcription-independent translocation of the Fas molecules from the Golgi to the cell surface [62]. Apoptotic signaling of these Fas and TRAIL receptors is generated through direct activation of the caspase cascade, leading to rapid execution of apoptosis [35, 62]. It is noteworthy that the TNF receptor superfamily is negatively controlled by its respective decoy receptors in a p53-independent manner [63, 64]. Finally, there is good evidence that p53 specifically regulates a number of redox enzymes, including PIG-3, PIG-6, and PIG-12, which represent homologues of quinone oxidoreductase, proline oxidase, and glutathione transferase, respectively. These enzymes promote the induction of apoptosis through the production of intracellular reactive oxygen intermediates (ROI) with subsequent mitochondrial damage and caspase activation [65]. This is consistent with the observations that p53-induced apoptosis is at least partially prevented by antioxidants such as *N*-acetylcysteine [66].

THE CONTROVERSY SURROUNDING p53 IN DRUG AND RADIATION RESISTANCE

Simple injury of actively proliferating cells seems to be insufficient for adequate action of most anticancer agents, but subsequent induction of apoptosis is regarded as a crucial step for successful cytostatic effect. Apoptosis represents an energy-dependent, genetically programmed process, and attenuations in the apoptotic pathways may consequently determine the therapeutic index of anticancer drugs and radiation. As mentioned above, p53 is an essential component of the apoptotic program induced by gamma irradiation and anticancer drugs in tumor cells. Mutations in p53 have been found to be associated with drug resistance *in vitro* as well as in animal models [67–69]. Epidemiological studies of p53 mutations and drug responsiveness yielded controversial results in a large variety of tumors treated with different regimens of cytostatic drugs [70–73]. Most trials showing an association between p53 mutations and chemotherapy resistance dealt with hematological malignancies. The influence of p53 on sensitivity to anticancer agents, however, is by no means clear in malignant tissues of non-hematological origin.

In solid tumors, apoptosis was not found to correlate consistently with overall cell killing following anticancer therapies. It was therefore suggested that p53-independent, delayed so-called mitotic cell death may be of pivotal importance in response to anticancer treatment and that the role of apoptosis has been overestimated by the often-applied short-term *in vitro* assays. The highly significant correlations of p53 mutations with drug resistance found in a number of investigations reflect short-term growth inhibition rather than effective cell killing [74]. In this context, it is also noteworthy that wild-type p53 can mediate growth arrest after DNA damage without inducing cell death. As long-term antitumor response seems to be more accurately assessed by the clonogenic assay, this method was proposed as being more suitable for studying cellular sensitivity to anticancer agents. Using clonogenic survival, p53 status was found to have no effect on the sensitivity of malignant cells to most anticancer drugs [74]. Moreover, loss of functional p53 was shown to confer sensitivity on DNA cross-linking drugs (e.g. cisplatin), probably through the lack of G1 checkpoint control and decreased cisplatin–DNA adduct repair [75, 76].

The dissociation of *in vitro* and *in vivo* results concerning the influence of p53 and other tumor suppressor genes on the cellular response to cytotoxic drugs and radiation, however, discloses that clonogenic assays are as unnatural as the short-term *in vitro* tests and highlights the importance of evaluating the role of these components in the context of the tumor microenvironment [77]. Thus, the issue of whether cancer therapy triggers apoptosis and whether p53 status is a valuable predictor of the final outcome of a cytotoxic treatment is far from settled.

The recent findings that wild-type p53 markedly suppresses the promoter activity of the multidrug resistance-associated protein (MRP) in cell lines established from solid tumors [78] add a further interesting point to this controversial discussion.

THE CONCEPT OF p53-BASED GENE THERAPY

Mutations of the tumor suppressor gene *p53* are the most common genetic alterations encountered in human cancers [79]. Its well-established physiological function and often decisive role in malignant transformation made p53 a logical candidate for the initial preclinical and clinical trials of gene therapeutical approaches. The re-expression of functional p53 in cells, in which the endogenous *p53* gene is deleted or mutated, has been demonstrated to restore a non-tumorigenic phenotype by suppressing tumor growth [80] and inducing apoptosis [81]. These findings were all the more astonishing since mixed mutant/wild-type p53 tetramers were believed to markedly diminish *trans*-regulation activity of p53 through a lack of or a significantly

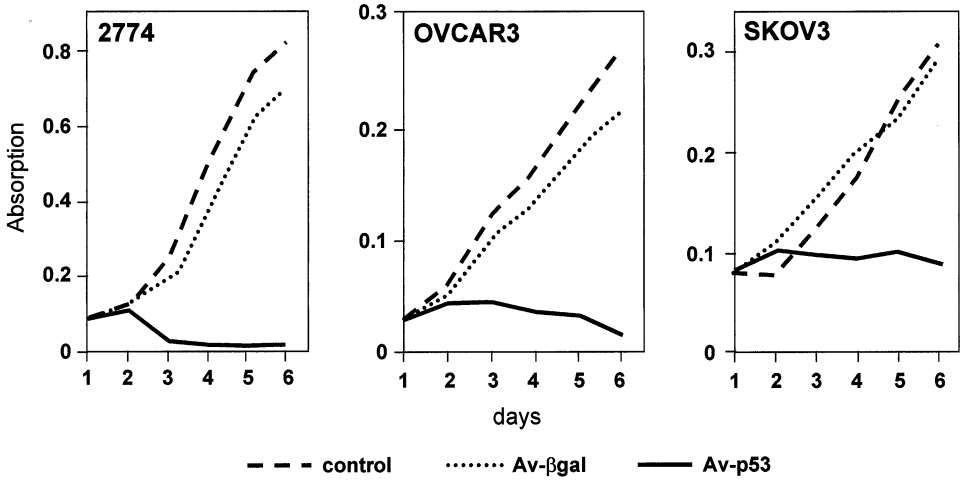

FIG. 2. The effect of Av-p53 and Av-βgal on ovarian cancer cell growth as compared to untreated controls. Cell number was evaluated using a colorimetric cell proliferation assay.

reduced binding affinity to consensus DNA. The most common mutations located in the DNA-binding domain, however, retain wild-type conformation and form stable complexes with authentic wild-type p53 [7]. The dominant negative hypothesis was further contradicted by Chen *et al.*, who showed that wild-type p53 was phenotypically dominant to a mutant p53 form at an equal gene dose ratio [80] and by a bicistronic expression system ensuring equal transcription of wild-type and mutant alleles [82]. Nevertheless, it is possible that certain, even though infrequent, missense mutations exist that are dominant to wild-type p53 [83]. Such "structural" mutations alter the conformation of p53, which then reacts with antibody PAb240 [84] and exhibit a loss of reactivity to PAb1620 and PAb246 [85]. Although it is tempting to speculate that an up-front characterization of the mutations in tumors could be valuable in predicting the efficacy of *p53* wild-type transduction, most clinical protocols so far do not consider this option.

An additional argument supporting the *in vivo* tumor suppressor-based gene therapy over other gene therapeutical approaches is the very probable lack of adverse effects on normal cells which are generally co-transfected by the usual non-tumor-specific vector systems.

The initial clinical trial of p53 gene therapy was conducted by Roth and colleagues using a retroviral vector to transduce wild-type *p53* into lung cancer [86]. In most subsequent study protocols, adenoviral vectors were favored due to certain advantages, including the high efficacy of transduction in non-dividing cells, the lack of insertional mutagenesis through extrachromosomal transfection, and the possibility of transferring large amounts of DNA. In contrast to the stable transfections obtained by retroviral vectors, gene transfer conducted by adenoviral vectors leads to a transient gene expression for a period of 14 to 21 days. Especially if adenoviral-based gene therapy is combined with other treatment modalities, this fact of unstable transfection should be taken into account in the establishment of a coordinated timetable for drug administrations. However, the potentially limiting step of adenoviral gene delivery is the high prevalence of neutralizing antibodies against adenoviruses in the general population, requiring an intratumoral or a locoregional vector administration. Since ovarian cancer generally remains confined to the abdominal cavity and dysfunction of *p53* has been reported in 55%–67% of advanced cases [87], this malignancy in our opinion represents an ideal disease for a p53-based intraperitoneal gene therapy. Preclinical transfection assays on established ovarian cancer cell lines gave promising results using a replication-deficient rAd-p53 [88]. Replication deficiency of this serotype 5-derived adenovirus was achieved by deleting the sequences for E-1A (encoding a potential oncogenic protein, which binds to cellular pRb) and for E-1B, which inactivates p53 through a physical association with the N-terminal region and promotes its degradation. The deleted viral regions are substituted with a cassette containing the wild-type *p53* cDNA driven by the cytomegalovirus (CMV) early enhancer and promoter [89]. As shown in Fig. 2, proliferation of cell lines 2774, OVCAR-3, and SKOV3 was significantly inhibited by rAd-p53 treatment when compared with cells treated with an adenovirus-carrying β-galactosidase reporter gene (rAd-β-gal). Most of this inhibitory effect was found to be due to increased apoptosis and to a much lesser extent to cell cycle arrest. These antiproliferative effects were also reproduced in nude mice inoculated with the above-mentioned ovarian cancer cells. Interestingly, in some experiments, animals treated with rAd-β-gal lived significantly longer than those treated with vehicle alone. This phenomenon was also observed in human breast cancer xenografts [90]. It was proposed that this unspecific antitumor effect was the result of the antiproliferative or cytotoxic action of cytokines such as interferons and TNF-α locally released by residual immunocompetent cells in nude mice. These findings suggest that immune cell activation induced by rAd-p53 could be responsible for a p53-independent bystander effect. A similar p53-mediated bystander effect is supposed through the antiangiogenic activity of wild-type p53, involving a reduced expression of vascular endothelial growth

factor and an increased expression of angiogenesis inhibitors (e.g. brain-specific BAI-1) in transduced cells [91, 92].

Of special note are the recently reported results that rAd-p53 mediates apoptosis in ovarian cancer cells independently of their endogenous p53 status [93]. These observations corroborate the hypothesis that intracellular concentrations of functional p53 above a certain threshold could be sufficient to induce apoptotic pathways, especially in cells with malignant phenotype. On the other hand, these results could possibly reflect an epigenetic transcriptional silencing of non-mutated *p53* gene in the investigated tumor cells. In addition, rAd-p53 treatment was shown to enhance the antiproliferative activity of platinum compounds and taxanes in ovarian cancer cells *in vitro* and in xenograft models [94]. The synergistic effect found for rAd-p53 and paclitaxel was demonstrated to be primarily due to an increased rAd-p53 transduction rate achieved after pretreatment of cancer cells with paclitaxel at doses lower than those required for microtubule condensation [95].

However, the most meaningful concern regarding p53-based gene therapy is related to the fact that transduction of wild-type *p53* in cancer cells does not necessarily induce apoptosis, but under some not fully defined circumstances may cause transient cell cycle arrest. In this regard, p53-generated up-regulation of $p21^{WAF1}$ seems to play a key role in influencing the response to DNA-damaging agents, including cytostatic drugs. Through cell cycle checkpoint restriction, $p21^{WAF1}$ can reduce the toxicity of cytostatic agents by increasing repair intervals or by coordinating DNA repair activity. High levels of $p21^{WAF1}$ determined in tumor specimens were found to be associated with chemoresistance and poor patient prognosis [96, 97]. Recent investigations revealed a growing body of evidence that $p21^{WAF1}$ represents a strong directly or indirectly acting inhibitor of apoptotic cell death [98–100].

It must be kept in mind that the possible role and impact of cell cycle arrest after *p53* gene delivery in malignant cells have to be clarified, and the question of whether the induction of apoptosis and in particular p53-dependent apoptosis is relevant for the anticancer effects of platinum compounds and taxanes remains unanswered. Nevertheless, the results of a large majority of retrospective studies revealing that *p53* mutations in ovarian cancers [101, 102] or the occurrence of anti-p53 auto-antibodies in the close tumor environment [103, 104] are associated with poor outcome of platinum-based chemotherapy, together with the preclinical data on wild-type *p53* transfection in ovarian cancer cells (especially in xenograft models), clearly advocated initiation of a phase 1 clinical trial [105]. This trial consisted of intraperitoneal administration of p53-based gene therapy combined with a systemic standard platinum/paclitaxel treatment in heavily pretreated patients with recurrent ovarian cancer. The evaluation of safety, which was the primary goal of this study, revealed that this treatment is feasible and well tolerated. Intraperitoneal catheter-related infections posed the limiting and most serious adverse event in this approach. A large ongoing phase 2/3 randomized multicenter study in patients with ovarian cancers carrying a p53 mutation will elucidate whether the addition of locoregional p53-based gene therapy to standard chemotherapy is superior to the control arm of carboplatin/paclitaxel.

GENE DELIVERY, THE RATE-LIMITING STEP IN GENE THERAPY

As mentioned above, adenoviral vector systems are favored in most gene therapy protocols. Their high infectability and capacity to transfer genes into non-dividing cells make adenoviruses especially suitable for the treatment of malignancies characterized by a low mitotic index. Adenoviral entry into target cells is the rate-limiting step of gene transfer. The initial interaction of adenovirus with the surface of the target cell has been shown to be mediated via a specific CAR. The knob of the adenoviral fiber is responsible for the viral attachment to this cell membrane receptor [106]. After initial binding, the viral particle achieves internalization via interaction with integrins of the $\alpha v\beta_3$ and $\alpha v\beta_5$ class [107]. Deficiency of either or both of these membranous molecule classes has been noted to confer relative resistance on adenoviral vectors [15, 16] (Fig. 3). Recently, heparan-sulfate-glycosaminoglycans expressed on cell surface were found to be involved in the binding of adenovirus to target cells. This finding is of major interest since heparin, which represents an analogue of these molecules, is able to decrease the infectability of adenoviruses [108].

It has been assumed that CAR is ubiquitously expressed, but there is a growing body of evidence suggesting that malignant transformed cells in particular can lose their ability to express this receptor. Marked differences in CAR expression in primary cultured ovarian cancer cells are depicted in Fig. 4. Moreover, in CAR-positive samples, immunohistochemical analyses revealed a pronounced heterogeneity for CAR staining, which could account for negative selection during adenovirus-based gene treatment.

There is no evidence that lost or reduced CAR expression is due to DNA mutations or rearrangements of the *CAR* gene [109]. Therefore, transcriptional regulation appears to play a pivotal role in modulating CAR expression. We are currently investigating whether or not hypermethylation in *CAR* promoter DNA accounts for gene silencing in CAR-negative ovarian cancer cells.

Paclitaxel was found to increase the adenoviral transduction rate *in vitro* [95] and in the SCID mouse model [110]. Preliminary data obtained in our laboratory indicate that paclitaxel induces the expression of CAR in a dose-dependent manner. The mechanisms by which paclitaxel influences transcription of the CAR gene remain to be elucidated.

Genetic modifications of the adenoviral fiber knob represent an interesting tool, not only to circumvent CAR deficiency but also to generate potentially more efficient

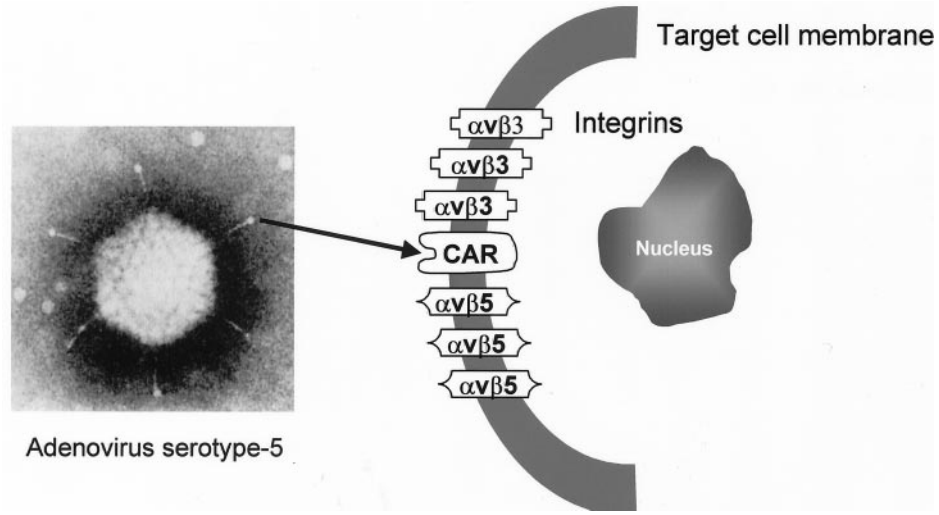

FIG. 3. Schematic illustration of adenoviral entry into a target cell. In a first step, *attachment* of the virion is achieved through the binding of the viral fiber knob to the specific CAR. The subsequent *internalization* is completed through an interaction of the adenovirus with integrins of the $\alpha v\beta_3$ and $\alpha v\beta_5$ class.

and tumor-specific vector systems. Recently, insertion of the Arg-Gly-Asp (RGD) peptide motif in the HI loop of the fiber knob protein was reported [111]. RGD insert binds with high affinity to integrins of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ class, which are abundantly expressed on the surface of a large number of malignant tumors. Viral targeting via growth factor receptors overexpressed in malignant tissue, such as epidermal growth factor receptor (EGF-R) or fibroblast growth factor receptor (FGF-R), have also been evoked [112, 113]. Vectors with growth factor antagonistic properties in addition to their gene delivery function could theoretically exhibit greater therapeutic efficiency.

In all gene therapeutical approaches, the expression of the specific receptor(s) in the target tissue corresponding to the applied vector system is essential for successful gene delivery. Therefore, determination of these target molecules prior to patient inclusion in gene therapeutical trials should be mandatory.

ADDITIONAL THERAPEUTICAL APPROACHES INVOLVING p53

ONYX-015 is an E-1B 55-kDa gene-deleted adenovirus that selectively replicates in and lyses human tumor cells with non-functional p53 [114]. Genuine adenoviruses infect quiescent cells and through the earlier-mentioned viral E-1A protein induce forced entry into S phase so that viral DNA replication can proceed. Oncoprotein E-1A, however, is also known to activate p53, which potentially prevents adenoviral replication and subsequent cell lysis. Nonetheless, this function is neutralized by E-1B 55-kDa protein, which binds and inactivates p53. Deletion of the E-1B 55-kDa gene therefore leads to a mutant virus that is unable to replicate in normal cells and in tumor cells with wild-type p53, but causes cell lysis and release of viral progeny in cells lacking functional p53. In addition, the selective replication and release of mutant viral particles may account for favorable intratumoral bystander effects. In a phase 1 trial, the feasibility of intraperitoneal ONYX-015 adenovirus administration was recently demonstrated in patients with recurrent ovarian cancer [115].

A second approach involving p53 is the reactivation of mutant p53 by pharmacological compounds. Several small synthetic molecules (300–500 Da) were recently identified which, with a hydrophobic end, fit into a hydrophobic pocket in p53 and, with a positive charge at the other end, attach to a negatively charged spot on p53. Like a molecular prothesis, these compounds enable mutant p53 to maintain a biologically active three-dimensional conformation with adequate gene *trans*-regulation functions. Furthermore, in an animal model, twice daily administration of

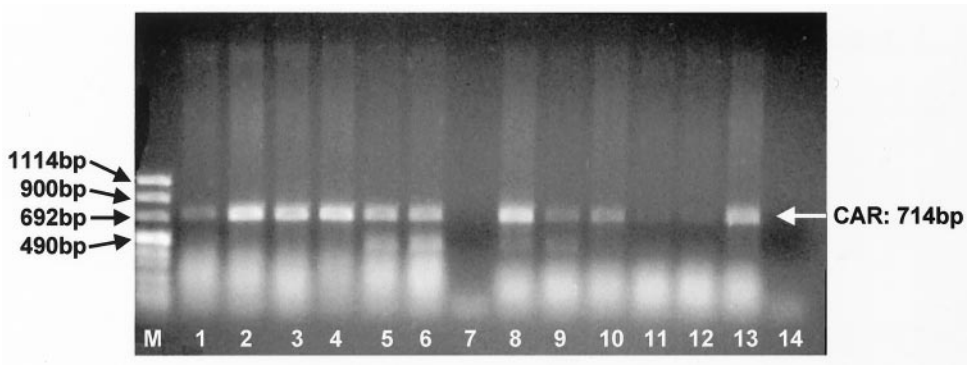

FIG. 4. Differences in the expression of CAR-specific 714-bp mRNA in ovarian cancer cells obtained from different patients.

these agents reduced tumor growth by more than 75% [116].

FUTURE PROSPECTS IN GENE THERAPY AND CONCLUSIONS

Cancer is a disease of the genome involving alterations in a multitude of different genes. It thus seems overly optimistic to think that correcting one molecular defect will be sufficient to exert a major impact on disease progression. After individual characterization of the specific genomic alterations, a "*multigene transfer*" with one single or different non-mutually competing vector systems should be more appropriate for abrogating the tumorigenic potential of targeted malignant tissue.

The *combination of gene therapy* with other treatment modalities represents one of the most promising options for the future. As exemplified for p53-based gene therapy, some therapeutic combinations worthy of special consideration are as follows. Additive or synergistic efficiency of wild-type p53 transduction combined with *cytotoxic drugs* has already been evidenced in preclinical and clinical studies. The underlying mechanism appears to be the restitution of functional apoptotic signaling, which is believed to play a crucial role in anticancer drug-induced cell killing. Recent *in vitro* data have shown that wild-type p53 expression in p53-mutated malignant cells sensitizes these cells to TNF-induced cytotoxicity by altering the cellular redox state [117]. These findings advocate further investigations in this field, including other recombinant human biologic response modifiers such as interferons and interleukins to evaluate a co-administration of these agents with p53-based gene therapy.

The addition of *immunotherapeutic* modalities to a wild-type p53 transfection is assumed to result in the induction of apoptotic cell death through different pathways. The feasibility of these combinations is currently under investigation.

Moreover, different *apoptotic signaling molecules*, which generally belong to ligands of the TNF receptor superfamily, can, besides their ability to induce apoptosis directly through activation of the cascade of caspases, also enhance p53-dependent apoptosis, but not cell cycle arrest. TRAIL (APO2L) is regarded as the best candidate for locoregional combination with p53 gene therapy due to its tumor-specific targeting, predominantly obtained through a high expression of TRAIL-protective decoy receptors on the surface of normal cells [63, 64]. Both hematopoietic and solid tumors illustrate the antiapoptotic nature of many growth factors by providing survival signals, not only for normal growth but also after cellular stress. It is conceivable that such growth factor-induced survival signals are a major mechanism of tumor resistance to apoptotic agents. Therefore, *neutralization of these growth factors* by available humanized monoclonal antibodies or antisense treatment very probably enhances the apoptotic and antitumorigenic power of p53 transduction. We and others have recently found that overexpression of HER-2/neu in ovarian cancer cells acts as an antiapoptotic factor [118], and we are currently evaluating the combination of p53 transfection with humanized anti-HER-2/neu antibodies in preclinical trials.

The development of new generations of *genetically modulated or new vector systems* allowing a higher gene transfer and a more tumor-specific targeting is mandatory for significant future advances in gene therapy. In this context, non-viral vectors such as liposomal systems are considered very attractive, especially due to their lack of immunogeneity, allowing systemic applications and reducing the risks of excessive immunological reactions against virus proteins.

In conclusion, correcting specific molecular defects responsible for the aberrant biological behaviour of cancer cells is a fascinating new approach in clinical cancer treatment. In view of the high frequency of p53 alterations in cancers and the central role of p53 in the regulation of growth and apoptosis, this appears to be an appealing target for gene therapy. Repairing one single, even important, gene will, however, probably not be sufficient to cure cancer.

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