No anti-apoptotic effects of single copies of mutant p53 genes in drug-treated tumor cells

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Some mutant forms of the p53 tumor suppressor have been documented to exert novel oncogenic functions including the increase of tumorigenicity, metastatic potential, genomic instability and therapy resistance of tumor cells. The latter has been suggested to be caused, primarily, by inhibition of apoptosis and, in part, through the activation of genes by mutant p53 whose products can counteract drug activities. Recently described in this context was the dUTPase, which may confer resistance to fluoropyrimidine drugs such as 5-fluorouracil (5-FU). We report here findings that call in question the existence of a direct anti-apoptotic effect of mutant p53. Wild-type p53-negative human fibroblasts, and Saos-2, H1299 and HCT116 tumor cells, treated with adriamycin, etoposide, cisplatin or 5-FU, failed to show apoptosis resistance when retrovirally bulk-infected to express the p53 mutants 175H or 273H at levels observed in naturally mutant p53-producing tumor cells. Furthermore, dUTPase gene expression was not stimulated by mutant p53, but instead by cellular events that involve DNA synthesis. We interpret the combined available data to suggest that much of the anti-apoptotic effect of mutant p53 is indirect and secondary to DNA-damaging and/or repair-interfering effects of these proteins. *Anti-Cancer Drugs* 15:679–688

Anti-Cancer Drugs 2004, **15**:679–688

Keywords: 5-fluorouracil, adriamycin, apoptosis, etoposide, cisplatin, drug resistance, mutant p53

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Sponsorship: This work was in part funded through the German Research Foundation (DFG; grant RO 2101/5-1).

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Received 12 April 2004 **Accepted** 30 April 2004

Introduction

Oncogene overproduction, hypoxia and DNA damage can provoke cell senescence and apoptosis through activation of the homotetrameric p53 transcriptional regulator and tumor suppressor [1]. Since these stresses are common in tumor cells, cancers have frequently lost p53 function. In notable contrast to many other tumor suppressors, however, the production of p53 is often not just stopped. Instead, one of a great number of full-length mutant forms of the protein is produced. These may act dominant-negatively by hetero-oligomerization, via the C-terminal tetramerization domain, with the wild-type p53 expressed from the remaining allele. However, dominance of a mutant is rarely complete and, consequently, the wild-type allele is often inactivated in the course of tumor progression. Apart from neutralizing wild-type p53, some mutants show a wide spectrum of pro-tumorigenic effects in cells that lack wild-type p53. For instance, mutant p53 may increase the tumorigenicity of cells [2], regulate genes [3,4], inhibit differentiation [5], increase mutation frequency [6] or metastatic potential [7] and cause genomic instability in a wild-type p53-null background [8,9]. The precise underlying mechanisms are still unknown. Recent work points to the possibility that at least some of these novel, wild-type p53-independent functions are less clearly set off from the dominant-negative function than initially thought: mutant p53 may not only hetero-oligomerize and interfere with wild-type p53, but also with the p63 and p73 members of the p53 family [10–12]. Other gain-of-function mechanisms may involve direct nucleic acid binding [13,14] and protein–protein interactions such as the binding and activation of topoisomerase I [9,15].

Many anti-cancer treatments provoke apoptosis that is not dependent on wild-type p53 [16] and the observation that mutant p53 may compromise such apoptosis has received much attention. For example, murine mutant p53 has been documented to confer to murine wild-type p53-null cells increased resistance to survival factor deprivation, irradiation and anti-cancer drugs [17]. Along the same line, certain human p53 mutants have been shown to inhibit the apoptosis of human p53-null osteosarcoma and lung adenocarcinoma cells treated with different chemotherapeutics [18,19]. However, many of the studies have made use of experimental designs that profit from strong overproduction of the transgene. Here we have studied the survival of cultures of several human wild-type p53-null cell types that were bulk-infected with retroviral vectors to constitutively express single

0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000136878.96680.f5

copies of mutant p53 genes, mimicking the presence of single mutated alleles in tumor cells.

Materials and methods

Chemicals and antibodies

The drugs adriamycin (ADR), etoposide, cisplatin, 5-fluorouracil (5-FU), thymidine and mimosine were from Sigma (St Louis, MO), as were G418, propidium iodide for DNA content analysis, glutaraldehyde and crystal violet for fixation and colony staining, and the β-actin monoclonal antibody. The p53 monoclonal antibody DO-1 was purchased from Calbiochem (San Diego, CA); the p53 monoclonal antibody DO-7 (Ab-12) and the cyclin E monoclonal Ab-1 were from Oncogene Research Products (Boston, MA). The monoclonal anti-dUTPase antibody was a gift of F. A. Grasser [20].

Cell culture, viruses and transfection

293GP retrovirus producer cells as well as O41, H1299 and Saos-2 cells were cultured in DMEM, and HCT116 cells and derivatives in McCoy's 5A medium, all were supplemented with 10% FCS and grown in a humidified 7% CO_2 atmosphere at 37°C. Retroviruses were harvested from the 293GP producer lines as previously described [21]. Cells were infected with titered virus stocks in the presence of 4 μg/ml polybrene (Sigma) for 4 h and virus-infected cells were selected in 400 μg/ml G418 at 48 h after infection for approximately 7 days. Exponentially growing cultures that had been out of selection for at least 2 days were drug-treated as outlined in the main text and figure legends. For transient transfection, exponentially growing cells in six-well dishes were incubated for 4 h with 0.4 μg of total DNA, of which 0.2 μg was empty vector, 0.1 μg was the green fluorescence protein expressing plasmid pEGFP-C3 (Clontech, Palo Alto, CA) and 0.1 μg was either wild-type or mutant p53-expressing plasmid (pCMV-wt53, pCMV-175H or pCMV-273H) or empty vector (pCMV-pA). As transfection reagent, effectene from Qiagen (Hilden, Germany) was employed according to the manufacturer's recommendation for six-well dishes. After 24 h, the cultures were either mock-treated or exposed to drug, as indicated in the main text and figure legends.

Flow cytometry analysis of DNA content and apoptosis

Cells were seeded in six-well dishes to approximately 30% confluency at 1 day before drug treatment. At the indicated time points, the cells on the dishes were harvested by trypsinization, and combined with the cells floating in the medium, fixed and stained with propidium iodide as described before [22]. The DNA fluorescence was measured with a Becton Dickinson FACScan (Bedford, MA) and the data were analyzed with CellQuest software from Becton.

Cell cycle synchronization and colony formation assays

Cells seeded at low confluency were grown for 1 day and were then exposed to thymidine (2 mM) for 12 h to establish a thymidine block. The cultures were washed once in prewarmed full medium and maintained in medium for another 12 h. To further synchronize the cultures, the cells were then incubated for 12 h with mimosine (400 μM; diluted from a 40 mM stock solution in 0.1 N NaOH), and the block was finally released through washes with prewarmed medium and the maintenance of the cultures in fully supplemented medium. The cell cycle distribution of the cultures was analyzed at different time points, after staining for DNA content, by flow cytometry with a Becton Dickinson FACScan and CellQuest software. For colony formation assays, 10^3 live cells (counted with a CASY cell counter) were seeded onto 10-cm dishes and grown for 24 h. The cultures were then incubated in the presence of drugs for 10 days. Colonies were washed with PBS, fixed with 1.25% glutaraldehyde for 20 min, washed again and stained with 1% crystal violet in PBS for 1 h at room temperature.

Immunoblot analysis

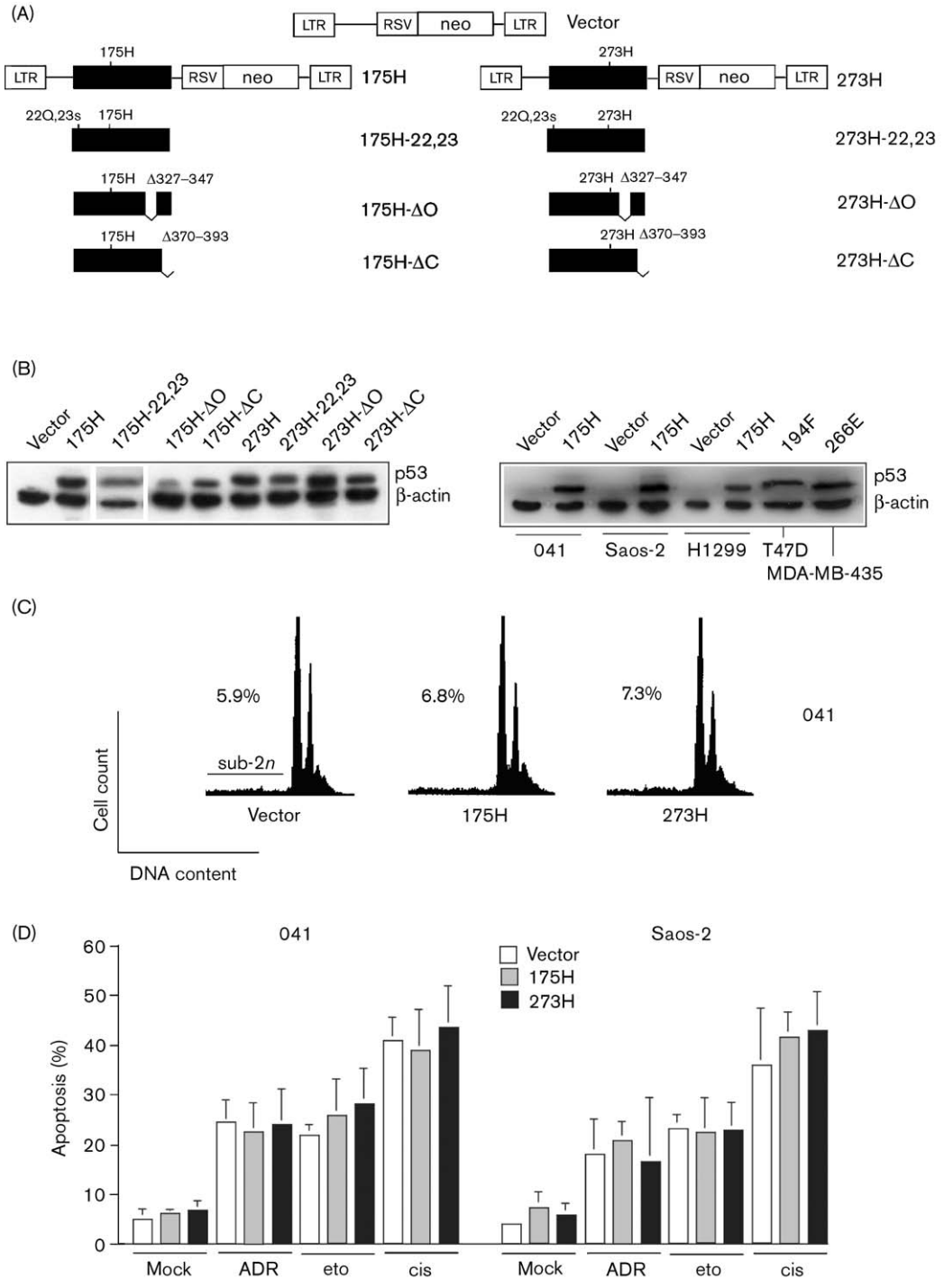
Cells from 10-cm dishes were lysed in 150 μl of a lysis buffer heated to 85°C, and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20% glycerol. Samples containing 15 μg of total cellular protein were subjected to 8 or 12% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Signals were detected upon overnight incubation of the membranes with anti-p53 (DO-1: 1:1000; DO-7: 1:500), anti-β-actin (1:5000), anti-cyclin E (1:500) or anti-dUTPase antibodies (1:20) followed by a final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, MA), performed as specified by the supplier.

Results

Apoptosis in p53-null cells versus mutant p53-expressing cells upon drug treatment

To gain further insight into the mechanisms of the anti-apoptotic gain-of-function by mutant p53, human immortal 041 (JML) fibroblasts, osteosarcoma Saos-2 cells and the lung adenocarcinoma H1299 cells, all p53-null, were infected with one of the retroviral vectors outlined in Fig. 1(A) at a multiplicity of infection (m.o.i.) of 1 c.f.u./cell or less that resulted in the transduction of a single transgene copy per infected cell. At 48 h after infection, the cultures were briefly (7 days) exposed to G418 to select for infected cells and selection was ceased several days prior to drug treatment. The result was cultures that contained either empty vector or produced one of the mutant p53 proteins at levels observed in the naturally mutant p53-expressing human tumor cell lines T47D and MDA-MB-435 (Fig. 1B). Cell proliferation

Fig. 1



Retroviral transduction of mutant p53 and apoptosis of drug-treated cells. (A) Retroviral vectors employed for the transduction of p53 mutants 175H and 273H, and variants thereof. LTR, long terminal repeat. RSV, Rous sarcoma virus long terminal repeat. Neo, Tn5-neomycin phosphotransferase gene. 22,23: substitution of p53 amino acid positions 22 and 23. ΔO and ΔC depict deletions in the oligomerization and regulatory domain, respectively. (B) Expression of the various transduced p53 mutants in 041 cells, and comparison of 175H expression levels in 041, Saos-2, and H1299 cells with the expression levels of 194F and 266E in the naturally p53-producing cell lines T47D and MDA-MB-435. Immunoblot analyses were preformed with the monoclonal anti-p53 antibodies DO-7 (left blot) and DO-1, and the β-actin antibody. (C) Flow cytometry DNA content profiles of untreated, exponentially growing 041 cultures containing either empty vector or the p53 mutants 175H and 273H. The percentages give the ratios of cells with a sub-2*n* DNA content indicative of apoptosis. (D) Cultures of cells transduced to express empty vector, 175H or 273H were either mock-treated or exposed to ADR (0.34 μM), etoposide (eto; 20 μM) or cisplatin (cis; 13 μM), and the percentages of apoptotic cells were determined by flow cytometry 36 h later.

studies failed to reveal significant growth differences between these cultures (data not shown). Furthermore, as exemplified in Fig. 1(C) for 041 cells infected with vector, 175H or 273H, analysis of the numbers of cells with a sub-*2n* DNA content indicative of apoptosis failed to detect large differences in the rates of apoptosis. Of note though, rather than resistance to apoptosis, as might have been expected in the case of anti-apoptotic gain-of-function, expression of the p53 mutants 175H and 273H consistently produced even slightly higher rates of apoptosis (Fig. 1C). Clearly, the p53 mutants had no obvious protective effects in any of the tested cell types. At least six independent infections of freshly thawed cultures were studied. When exponentially proliferating cultures of 041, Saos-2 or H1299 cells, infected with one of the retroviral constructs, were either mock-treated or exposed to ADR (0.34 μM), etoposide (20 μM) or cisplatin (13 μM), and were analyzed 36 h later, it became apparent that each drug was able to provoke apoptotic death as expected (Fig. 1D, white bars), indicating that neither the infection procedure nor the temporary G418 exposure had compromised the ability of the cells to respond to the drugs. However, and despite much effort, no significant differences in the apoptotic responses between controls and mutant p53-expressing cultures could be detected after 36 h (Fig. 1D) and up to 72 h of drug treatment (data not shown). Similarly, variants of the mutants that were further mutated in the transactivation, oligomerization or regulatory domain (see Fig. 1A) also failed to affect the drug-induced apoptosis. We thus conclude, in contrast to many previous reports (see discussion), that the p53 mutants 175H and 273H do not, under the above detailed conditions, exert anti-apoptotic functions in drug-treated human wild-type p53-null 041, Saos-2 and H1299 cells.

Apoptosis in transiently transfected cells

Retroviral infection at low m.o.i. (to ensure single copy transgene expression) required the temporary selection with the antibiotic G418. Since it could not be excluded that G418 selection can inhibit putative, mutant p53-dependent anti-apoptotic pathways, we also performed apoptosis studies on cultures that had been transiently transfected with expression plasmids, then drug-treated to induce apoptosis and finally analyzed by flow cytometry. When Saos-2 cultures were transfected with empty vector or vector expressing either wild-type p53, 175H or 273H, plus a vector producing green fluorescence protein for the identification of transfected cells in flow cytometry, expression of the transgenes to approximately equal levels could be documented (Fig. 2A). The cultures were then mock-treated or exposed to etoposide (20 μM) for 36 h. As expected, wild-type p53 reduced the number of surviving transfected cells even in the absence of drug, while mutant p53 produced a similar number of transfected cells compared to empty vector (Fig. 2B), reflecting the well-documented pro-apoptotic function of wild-type, but not mutant, p53 in Saos-2 cells even in the absence of damage. In the cultures exposed to the drug, the number of surviving wild-type p53-transfected cells was further reduced relative to the vector-transfected controls. In contrast, the number of surviving transfected cells containing empty vector or mutant p53 decreased moderately and by similar rates, indicating that mutant p53 failed to rescue transfected cells from death. Figure 2(C) summarizes the quantification of surviving transfected cells after drug treatment and confirms the results obtained with the retroviral transductions showing that 175H and 273H exert no anti-apoptotic function in our experimental setting.

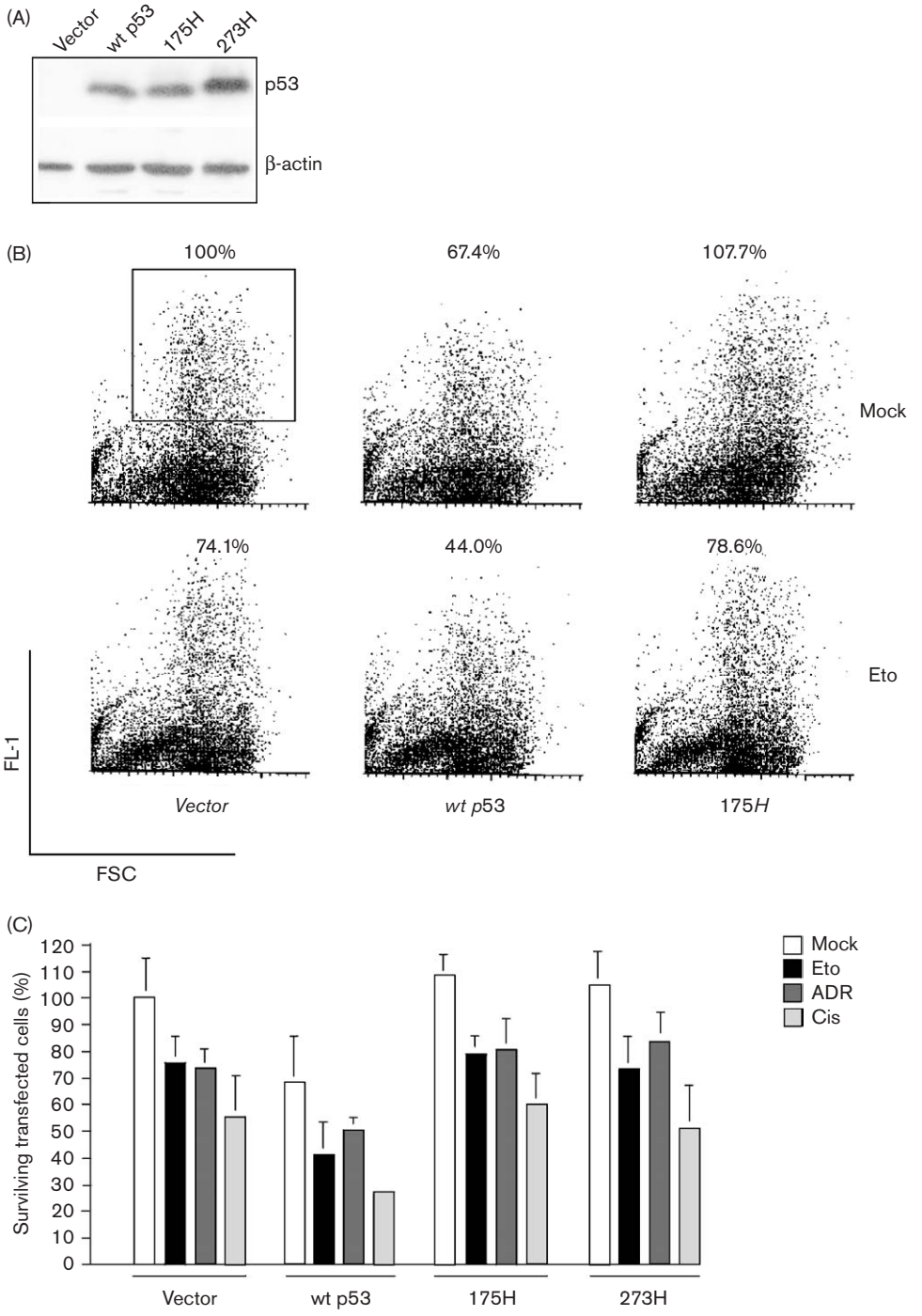
Human HCT116 colon and H1299 lung adenocarcinoma cells

HCT116 is a poorly differentiated, growth factor-insensitive cell line exhibiting the microsatellite instability (MIN) phenotype due to deficiency for hMLH1, the human homolog of the bacterial Mut L protein and essential factor of DNA mismatch repair. HCT116 cells possess an intact p53/p21Waf1/pRb tumor suppressor pathway, and an isogenic cell line is available in which p53 had been knocked out by targeted homologous recombination [23]. On the basis of these HCT116 $p53^{-/-}$ cells we generated bulk-infected cultures of cells with single copies of either empty vector, 175H or 273H, and with the p53 mutants being expressed at levels comparable to the wild-type p53 levels in HCT116 cells (Fig. 3A). There were no significant differences in the population doubling times and FACS profiles between the cultures (not shown). ADR (0.34 μM), cisplatin (13 μM) or the DNA/RNA-damaging drug 5-FU (375 μM) all provoked apoptosis, as exemplified for the 48 h timepoint in Fig. 3(B), yet no differences in cell death response could be detected between cultures with or without mutant p53. This, again, indicates the lack of an obvious anti-apoptotic gain-of-function of 175H and 273H.

Recent work by others [24] has pointed to the existence in several tumor cell lines, including H1299, of a more subtle anti-apoptotic effect by 175H in the presence of 5-FU. The relative resistance to 5-FU was suggested to be the result of activation of the dUTPase gene expression by mutant p53, since 5-FU inhibits thymidylate synthase and thereby favors mis-incorporation into DNA of uridine from dUTP, while dUTPase lowers dUTP levels. Along the same line, we have documented previously that, in turn, knockdown of dUTPase by antisense expression can result in apoptosis [25]. One might thus envision that upregulation of dUTPase by mutant p53 is anti-apoptotic.

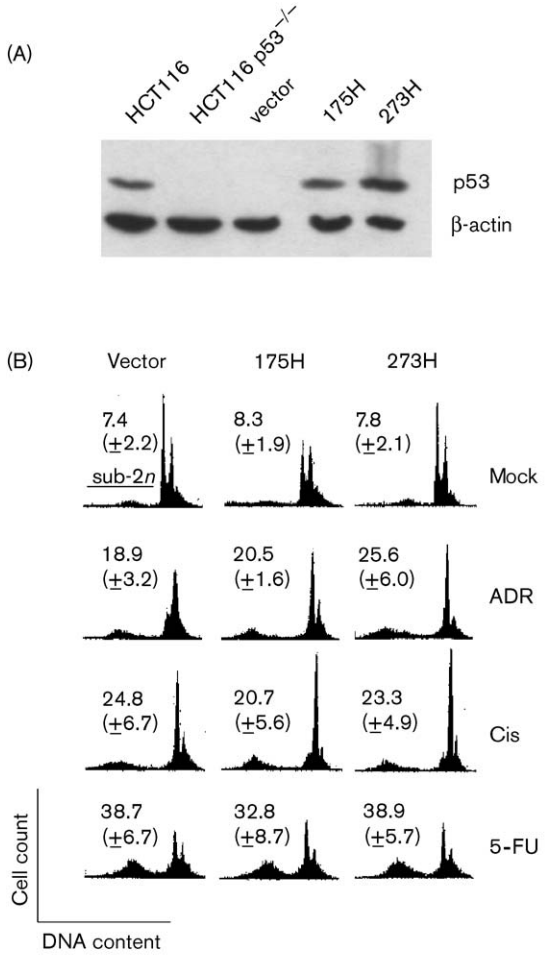
When we studied dUTPase expression by RT-PCR in our H1299 cultures, we failed to observe a correlation between mutant p53 expression and a rise in dUTPase

Fig. 2



Apoptosis in transiently transfected cells. (A) Saos-2 cultures were transiently transfected to express empty vector, wild-type p53, 175H or 273H as detailed in the main text and in Materials and methods. The steady-state levels of p53 and β-actin were examined by immunoblotting using the anti-p53 antibody DO-1 and the β-actin monoclonal. (B) Co-transfection of Saos-2 cultures with vector-only or the p53 expression plasmids plus a plasmid producing green fluorescence protein allowed the detection of surviving transfected cells (window) as fluorescence (FL-1)-positive cells with high forward scatter (FSC). The transfected cultures were either mock-treated or treated with etoposide (eto; 20 μM). The numbers of surviving transfected cells in vector-transfected, mock-treated cultures were arbitrarily set as 100%. (C) Quantification of the numbers of surviving transfected cells upon treatment relative to that number in mock-treated vector-expressing cultures (set as 100%). Cultures were variably transfected to produce wild-type p53, 175H or 273H, and were mock-treated or exposed to etoposide (20 μM), ADR (0.34 μM) or cisplatin (cis; 13 μM) for 36 h.

Fig. 3



Drug-induced apoptosis in HCT116 cells and derivatives. (A) Immunoblot analysis documenting the steady-state expression of wild-type p53 in the parental HCT116 cells, the lack of p53 expression in HCT116 $p53^{-/-}$ cells and in HCT116 $p53^{-/-}$ cells infected with empty retroviral vector (vector), and the 175H and 273H expression in HCT116 $p53^{-/-}$ cells infected with the respective viruses. The antibodies were anti-p53 DO-1 and anti-β-actin. (B) Flow cytometry analysis of the DNA content of HCT116 $p53^{-/-}$ cultures expressing empty vector or mutant p53 and treated with ADR (0.34 μM), cisplatin (cis; 13 μM) or 5-FU (375 μM) for 48 h. The numbers indicate the percentages of apoptotic cells; SDs of five experiments are given in parentheses.

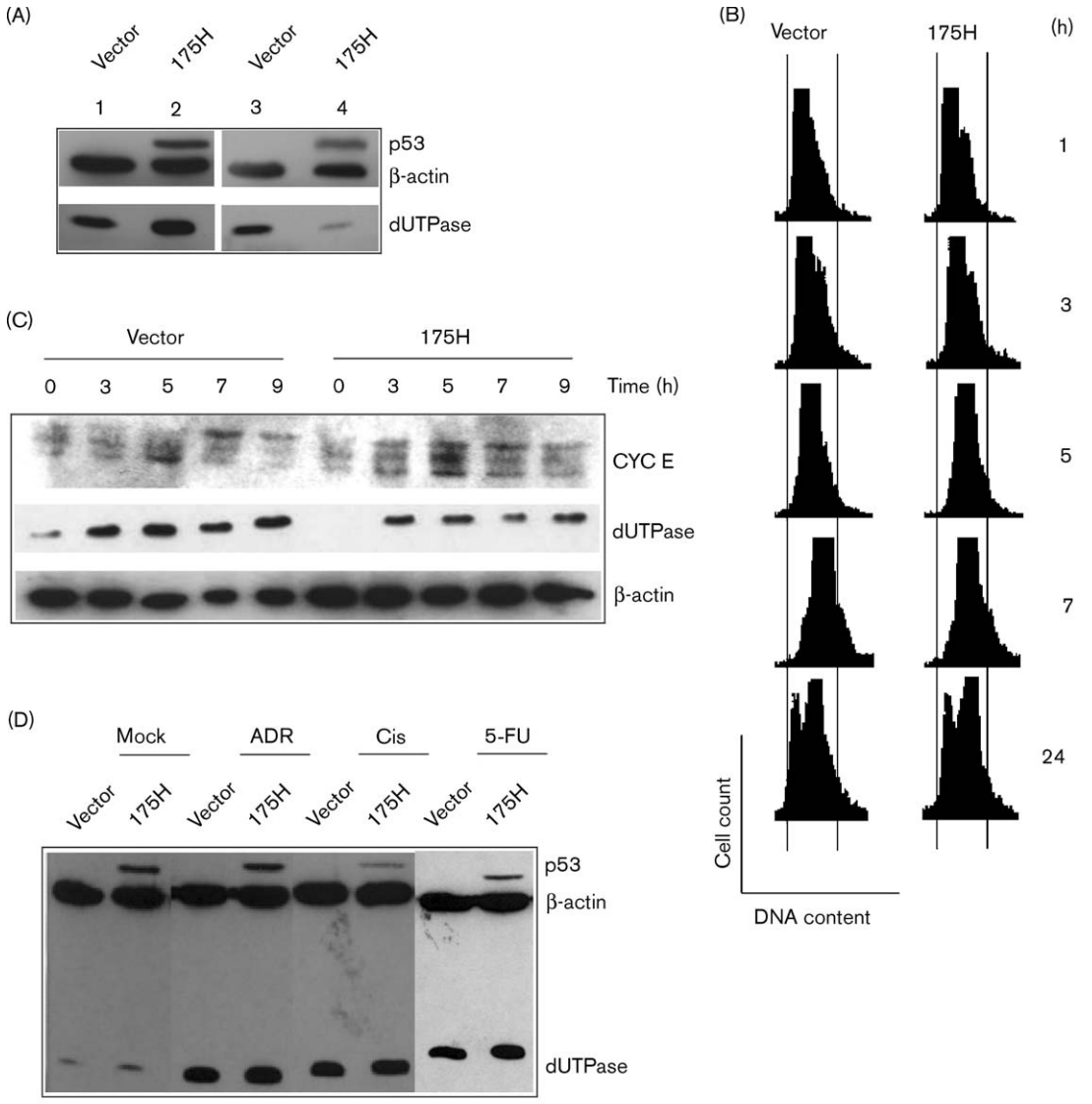
transcripts (not shown); rather, despite of constant expression levels of GAPDH control transcripts, the dUTPase transcript levels were highly variable, independent of the presence of mutant p53. Similar observations were made when dUTPase protein levels were analyzed in immunoblots (Fig. 4A). Here, too, the dUTPase quantities varied between different cultures (lanes 1–4) and independently of p53. Since the dUTPase was recently reported to be expressed in a cell cycle dependent manner primarily during S phase [26], we entertained the possibility that the differences in dUTPase expression between the different cultures reflect differences in the numbers of cells with DNA synthesis.

When exponentially growing HCT116 and H1299 cultures were subjected to a thymidine/mimosine cell cycle block (see Materials and methods for detail) and were then released from this block, flow cytometry revealed the coordinated passage of the cells through the cell cycle within 24 h (Fig. 4B, and data not shown). At approximately 5 h after the release, the cells entered S phase, as could also be documented by the accumulation of cyclin E and the subsequent change in cyclin E phosphorylation pattern (Fig. 4C). Accumulation of dUTPase paralleled the progression of the cultures through S phase, in accord with previous results showing that the expression of the enzyme is cell cycle dependent [26]. Furthermore, there was no notable difference in the synchronization of the cell cycle and in dUTPase accumulation whether the cells contained mutant p53 or vector only, demonstrating that mutant p53 fails to affect dUTPase expression in S phase (Fig. 4C). However, and notably, when asynchronously growing cultures were treated with DNA-damaging drugs, dUTPase levels rose significantly regardless of the fact that the cells then accumulated at the G_2/M border (upon ADR and cisplatin treatment) rather than in S phase. Thus, dUTPase expression is not only stimulated during DNA replication in S phase, but also following DNA damage induced by ADR, cisplatin or 5-FU treatment (Fig. 4D). Combined, these data indicate that dUTPase expression is not directly regulated by mutant p53 and that dUTPase is thus unlikely to be a mediator of an anti-apoptotic gain-of-function of mutant p53. This conclusion was further supported by colony formation assays. When H1299 or HCT116 $p53^{-/-}$ cells were infected with single copies of empty vector, 175H or 273H, were then seeded onto 10-cm dishes to produce similar numbers of colonies, and were maintained in the presence or absence of increasing doses of 5-FU for 10 days, there was no difference in the reduction of colony numbers (Fig. 5 and data not shown), again documenting the lack of a survival advantage in the presence of mutant p53.

Discussion

Wild-type p53-independent, oncogenic functions of mutant forms of p53 have been clearly established by a large number of studies (for review, see [27]), yet the precise nature of these functions is still elusive. In particular it seems to us that the existence of a direct 'anti-apoptotic' gain-of-function in the presence of therapeutic drugs, as well as the possible mechanism that might underlie such an effect, is unclear. On the one hand, several reports have documented increased therapy resistance of p53-null tumor cell lines under experimental conditions that relied on transient or stable

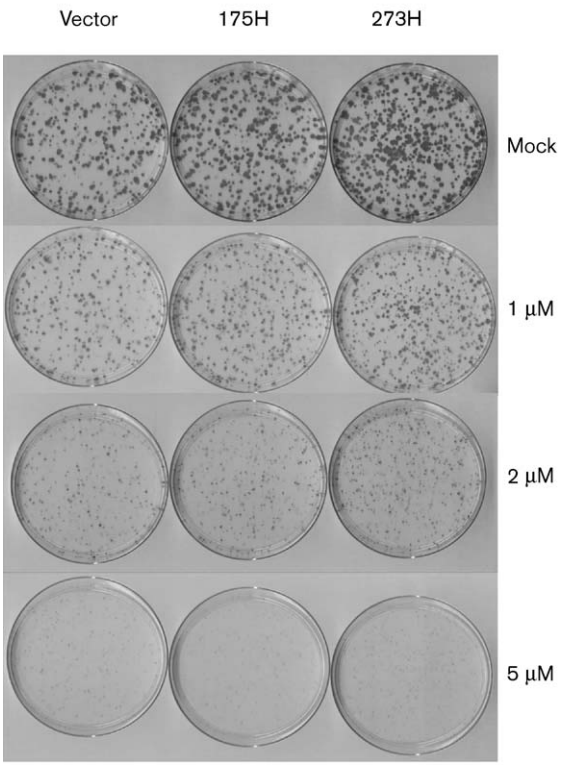
Fig. 4



dUTPase steady-state expression is regulated by DNA synthesis and damage rather than by mutant p53. (A) dUTPase levels were highly variable in the four separate H1299 cell cultures expressing vector-only or 175H, independently of mutant p53. (B) Example of a cell cycle synchronization of H1299 cultures infected with empty vector or 175H-vector, through application of a transient thymidine/mimosine block as detailed in Materials and methods. Fractions of flow cytometry profiles are shown. The two vertical lines in each profile indicate the beginning (G_1 phase) and end (G_2/M phase) of a cycle. The time after the release from the thymidine/mimosine block is given in hours. Note that the cells move through S phase between 3 and 7 h. (C) Immunoblot analysis of H1299 cells expressing vector or 175H, at different times after the release from the cell cycle block. Note the changes in the level, and the phosphorylation-induced shift, of cyclin (cyc) E at approximately 5 h, characteristic for cells moving through S phase. The patterns of dUTPase expression in the vector and 175H cultures were similar. (D) Immunoblot analysis of vector- or 175H-expressing cells either mock-treated or treated with ADR (0.34 μM), cisplatin (cis; 13 μM) or 5-FU (375 μM). Signals were detected with the p53 (DO-1), β-actin and dUTPase monoclonal antibodies.

mutant p53 overproduction. For example, murine myeloblastic cells showed higher resistance to γ-radiation, ADR and cisplatin [17], Saos-2 cells to cisplatin [18], Saos-2 or H1299 cells to etoposide or cisplatin [19], and *ras*-transformed $p53^{-/-}$ mouse embryo fibroblasts to γ-radiation, cisplatin and other treatments [28]. Since cytotoxicity of anti-cancer drugs seems to be primarily mediated through apoptosis, and strong transactivation of the multidrug resistance gene 1 (MDR-1) does not appear to be a general phenomenon in mt p53-expressing cells, therapy resistance and apoptosis resistance are usually considered to be synonymous. On the other hand, previous studies have failed to establish correlations between the expression of mutant p53 and anti-apoptotic genes [29]. Moreover, several investigators have failed to observe a correlation between p53 mutation and chemo-

Fig. 5



Colony formation assay with H1299 cells infected with empty vector or with a mutant p53-transducing retrovirus. Live cells (10^3) were seeded on 10-cm dishes and were either mock-treated or exposed to increasing doses of 5-FU for 10 days. Colonies were then fixed and stained with crystal violet.

or radioresistance [30,31]. For example, work by Cote *et al.* [31] has suggested that patients with transitional cell carcinoma of the bladder, surprisingly, benefit from adjuvant ADR and cisplatin chemotherapy only when their tumors express mutant p53. That mutant p53 can indeed exert pro-apoptotic function in certain cell types has been documented previously by us [21] and others [32]. Here we report that some of the very same tumor cell types in which mutant p53 has previously been shown to exhibit anti-apoptotic gain-of-function upon transient and stable transfections [19,33] are non-responsive following retroviral transduction or transfection of the same mutants. Why are the results contradictory and how can the contradictions be resolved?

Some discrepancy is likely to reflect methodological differences between the studies. Blandino *et al.* [19] have documented that H1299 cells are significantly protected from etoposide-induced apoptosis when overproducing the p53 mutant 175H and, to a lesser extent by, 273H. Furthermore, the authors have reported that 175H and 273H can partially protect H1299 cells from the toxic effects of low doses of cisplatin. They had either transiently or stably transfected cells and had gated the high p53 expressors in flow cytometry to compare the numbers of apoptotic cells between drug-treated and control-treated populations. Wang *et al.* have reported that transfection and overproduction of 175H and 273H in Saos-2 cells can confer relative resistance to cisplatin [18]. In contrast, we were unable to detect any anti-apoptotic effects of the p53 mutants 175H and 273H in Saos-2, H1299 and other p53-null cell types treated with ADR, etoposide or cisplatin. One explanation for these discordant results could be that the mutant p53 expression levels in the former studies were higher compared to the levels achieved here, as we have used retroviral transduction of single copy genes to mimic the presence of single mutant p53 alleles or have used a transfection procedure that employs much smaller DNA quantities. In accord with this explanation, Blandino *et al.* [19] have failed to observe a protective effect in the low expressor subpopulations of their 175H and 273H transfected cultures. Thus, high mutant p53 levels may be protective to some degree, while low levels may not.

How might high levels of mutant p53 protect from drug-induced apoptosis? Several mechanisms may be envisioned. For example, mutant p53 may be able to induce DNA damage and/or affect repair processes, perhaps through the transcriptional stimulation of c-*myc* [13], which has been reported to be able to induce DNA damage via the induction of reactive oxygen species [34]. Previous work has furthermore shown that the spontaneous and induced mutation frequency at the autosomal thymidine kinase locus in lymphoblastoid cells increases to a greater extent in cells harboring mutant p53 than in p53-null cells [35,36]. Moreover, mutant p53 can foster genomic instability in p53-deficient cells, perhaps in part through the induction of centrosome abnormalities [33]. Finally, as has recently been shown for 273H in Saos-2 cells, mutant p53 can interact with and activate topoisomerase I [9,15], an interaction that is constitutive while the interaction between wild-type p53 and topoisomerase I is strictly regulated during DNA damage repair. The constitutive interaction between mutant p53 and active topoisomerase I may result in DNA double-strand breaks, which alone or in conjunction with centrosome abnormalities may not only favor genomic instability, but activate a (wild-type p53-independent) cell cycle checkpoint. Active checkpoints can suppress apoptotic responses (e.g. [23]) and might thus mimic an anti-apoptotic gain-of-function of mutant p53.

The eventual appearance of genomically instable cells in the presence of mutant p53 indicates that cell cycle checkpoint activation by mutant p53, if it occurs at all, is not sustained and that some cells eventually escape. Such cells might then experience apoptosis, unless genetic alterations allow them to escape from that fate as well. Thus, it is conceivable that newly induced, high levels of

mutant p53 (as are likely present in high-expressor populations of transfected cell cultures) can acutely suppress drug-induced apoptosis and that permanent expression of (perhaps lower levels of) mutant p53 may eventually select for genetically altered cells that are resistant to apoptosis. In cells with more moderate mutant p53 levels, however, which had not been selected for a long time, mutant p53 may not significantly affect drug-induced apoptosis at all. This view is supported by very recent results [28] concluding that the mutant p53-mediated cellular resistance to chemotherapy could be the result of a selection process. Combined, we interpret the available data to suggest that much of the anti-apoptotic effects of mutant p53 are indirect and secondary to other, perhaps DNA-damaging or repair-interfering effects of these proteins.

How does this suggestion relate to other known gain-of-function effects of mutant p53? Although not constituting a classical transcription factor, expression of mutant p53 has been reported to entail numerous alterations in the gene expression profiles of cells [3,4,27], with one affected gene being that for the dUTPase. Pugacheva *et al.* have reported that transient overproduction of 175H, but not 273H, from an inducible promoter can stimulate dUTPase transcript expression in H1299 cells and have suggested this to confer resistance to apoptosis by 5-FU. In our hands, 175H and 273H constitutively produced from a retroviral vector failed to stimulate dUTPase expression at both the transcript and protein level, and to confer resistance to 5-FU. Rather, dUTPase expression raised during S phase, as has been reported before [26], and was furthermore stimulated upon DNA damage, independent of mutant p53 expression. This is again compatible with the suggestion discussed above that transient overproduction of mutant p53 may (directly or indirectly) cause DNA damage, and that (some) gene expression and anti-apoptotic effects are secondary. Clearly, this issue requires further study.

Conclusion

Mutant forms of the p53 tumor suppressor which had previously been reported to be able to act anti-apoptotic in drug-exposed tumor cells and when overexpressed, fail to show anti-apoptotic effects in the same tumor cell lines when expressed from single gene copies. A direct anti-apoptotic function of mutant p53 in these widely used cell systems can thus not be confirmed. Furthermore, expression of the dUTPase which is thought to confer resistance to fluoropyrimidine drugs is not directly stimulated by mutant p53 as reported before, but instead is stimulated by DNA synthesis. dUTPase overexpression upon transient overproduction of mutant p53 may therefore be the result of DNA damage, which may entail the activation of a cell cycle checkpoint and some apoptosis inhibition as a secondary effect.

Acknowledgments

We thank Dr B. Vogelstein (Johns Hopkins University, Baltimore, MD) for the cell lines HCT116 and HCT116 $p53^{-/-}$, and Dr F. A. Grasser (Department of Virology, Homburg, Germany) for the anti-dUTPase monoclonal antibody.

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