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Endogenous p21$^{WAF1/CIP1}$ status predicts the response of human tumor cells to wild-type p53 and p21$^{WAF1/CIP1}$ overexpression

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Expression of exogenous wild-type (wt) p53 protein can suppress the growth and/or induce apoptosis in different tumor cells. The effect of exogenous p21$^{WAF1/CIP1}$ expression is more controversial: while it can induce apoptosis in some cells, it can protect against p53-mediated apoptosis in others. We used adenoviral vectors to introduce p53 and p21$^{WAF1/CIP1}$ genes into human tumor cell lines with different p53 and/or p21$^{WAF1/CIP1}$ status. The cell growth inhibition and the induction of apoptosis were measured. Overexpression of wt p53 induced more efficient growth inhibition and apoptosis in SW 620 (mutant p53) and HeLa (inactivated p53 protein) than in MCF-7 (wt p53) and CaCo-2 cell line, which was the most resistant to p53 overexpression despite the p53 mutation. Unlike HeLa and SW 620 cells, the basal p21 protein level was readily detected in CaCo-2 and MCF-7 cells. Overexpression of p21$^{WAF1/CIP1}$ gene induced somewhat less pronounced growth inhibition of all cell lines tested, but it also induced apoptosis in HeLa and SW 620 cells. These results suggest that the basal, but not the inducible, levels of p21$^{WAF1/CIP1}$ protein in tumor cells could protect from p53-mediated apoptosis. On the other hand, overexpression of p21$^{WAF1/CIP1}$ gene itself can induce apoptosis in cells with no basal p21$^{WAF1/CIP1}$ protein level. Possible mechanisms of the differential response to these genes are discussed.

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In spite of extraordinary advances in our understanding of cancer, the key molecular changes in the multistep progression of cancer are still unknown. A better understanding of this process will eventually produce better diagnostic and prognostic tools and more rational and effective therapies.[1,2] Although numerous genetic alterations have been detected in cancer, activation of oncogenes and inactivation of cell cycle regulators (e.g. tumor suppressor genes) are known to play a critical role in the progression of the disease. Tumor suppressor genes participate in critical cell functions including signal transduction, gene transcription and control of cell proliferation or cell death. Loss or mutation of both or, sometimes even a single allele, of these genes usually contributes to the malignant phenotype.[3] Theoretically, substitution of mutated or deleted tumor suppressor gene with the functional copy could restore normal growth and proliferation pathway.[4]

The p53 tumor suppressor gene is one of the most commonly mutated genes in human cancer.[5] It encodes a nuclear phosphoprotein that, as a transcription factor, regulates the synthesis of gene products involved in growth arrest, DNA repair, apoptosis and the inhibition of angiogenesis. Therefore, it plays a critical role in mediating cell cycle arrest and/or apoptosis following exposure to stress stimuli.[6,7] Reconstitution of normal p53 expression in tumor cells can lead to suppression of cell growth and cell death. Previous studies have demonstrated that introduction of wild-type (wt) p53 gene by viral-mediated delivery can suppress growth in a number of human cancer cell lines *in vitro* and *in vivo*. The general conclusion from these studies is that the introduction of wt p53 into tumor cells caused specific inhibition only in those cells that contained mutated or null p53. Both, normal and tumor cells retaining p53 function were not so strongly affected, unless other factors influenced p53 function (such as human papillomavirus, HPV). The overexpression of exogenous wt p53 in most cases induced apoptosis only in the p53^{mut} or p53^{null} cells.[8,9] However, the results published so far suggest that there is no clear-cut rationale for the response of a tumor to tumor suppressor gene transfer, and the mechanism by which p53 mediates apoptosis is not well understood. For instance, there are studies that demonstrate that the presence of either HPV or mutant p53 inhibit apoptosis after wt p53 overexpression,[10] that the endogenous p53 status does not correlate with the induction of apoptosis,[11,12] or that it does not activate it at all.[13] The effects of overexpression of p53-inducible gene p21 (WAF1/CIP1) are even more controversial. In some tumor cells, induction of p21 expression correlates with apoptosis,[14,15] while in some others induction of p21 is only associated

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with growth inhibition.[16–18] Moreover, there are studies that demonstrate the protective role of p21 gene against p53-mediated apoptosis and indicate that the presence of p21 confers a survival advantage.[19–21]

Most features of cancer cell biology have been tentatively exploited for either conventional or gene therapy.[22] Since, the end point of virtually all these methods is the induction of apoptosis, it is of great importance to better understand the mechanisms of cell cycle and/or apoptosis regulation, especially those responsible for the divergent responses of cells to exogenous wt p53 and p21 expression. For this purpose, we compared the efficacy of the exogenous wt p53 gene expression with that of p21 gene on growth inhibition and induction of apoptosis in four human tumor cell lines, which have different intrinsic p53 protein status (wt, HPV-inactivated or mutant). We introduced the two genes via recombinant adenoviral vectors (Ad5CMV-p53 and Ad5CMV-p21). In the light of the possibility that the p21 gene could have a protective role against p53-mediated apoptosis, we also examined the effects of these two vectors in regard to the intrinsic p21 status. We were especially interested in the exogenous wt p53 overexpression effect in CaCo-2 cell line, which, according to Djelloul et al[23] contains one deleted and one mutant p53 allele. However, CaCo-2 cell line differentiates spontaneously after reaching confluence and p21 expression is induced during this process in a p53-independent manner.[24] The intrinsic p21 protein level could thus inhibit exogenous wt p53-induced apoptosis in this cell line, regardless of the mutation in the p53 gene.

Methods

Cell lines

Four human tumor cell lines having different p53 status were used: HeLa (cervical carcinoma), which express wt p53, but the protein product is HPV-inactivated; MCF-7 (breast carcinoma), which express wt p53; SW 620 (human colon carcinoma), which overexpress mutated p53; and CaCo-2 (human colon carcinoma), which have one mutant and one deleted allele. CaCo-2 cells are poorly tumorigenic in nude mice and spontaneously differentiate in culture.[23] All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Recombinant adenoviral vectors

Recombinant adenoviral vectors Ad5CMV-p53 and Ad5CMV-p21 (Introgen Therapeutics, Inc., USA) contain the CMV promoter, wt p53 and p21 cDNA, SV 40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5, as described earlier.[25] As a control vector dl 312 was used. Viral vectors were propagated and titrated in 293 cells. Cells were harvested 36–40 hours after infection, pelleted, resuspended in phosphate-buffered saline and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted and stored at −80°C.

Cell growth assay in vitro

The cells were plated in triplicates at a density of 1×10^4 cells/ml in 24-well plates 24 hours before viral infection. Infection was carried out by adding the virus to the cell monolayers in 200 μl DMEM supplemented with 2% FBS. The cells were incubated at 37°C for 60 minutes. The complete medium (DMEM with 10% FBS) was added and the cells were incubated for the desired length of time. Cells were harvested every 2 days and counted manually; their viability was determined by trypan blue exclusion method. The cell growth was expressed as a percentage of viable infected cells, in relation to the number of viable control (uninfected) cells, which was expressed as 100%.

Semiquantitative RT-PCR

Total RNA was prepared from either noninfected or dl-312-, Ad5CMV-p53- and Ad5CMV-p21-infected cells by using RNAzol™ B kit (Biogenesis, England). RNA (1 μg) was treated with RNAse-free DNAse (Roche) for 30 minutes at 25°C, followed by 10 minutes at 75°C, and subsequently used as a template for cDNA synthesis in a 20 μl reaction. The reaction mixture contained 0.5 mM dNTPs (Roche), 0.5 μg oligo d(T)18 primers (New England Biolabs Inc.), 10 mM DTT, 1 × RT buffer and 200 U™ RNase H^- Reverse Transcriptase (Gibco). Reaction mixtures were incubated for 1 hour at 45°C, heated to 70°C for 15 minutes, and to 94°C for 15 minutes. A volume of 80 μl of dH_2O were added to the reactions and 3 μl of the dilution was used as template for PCR. The cDNA was stored at −20°C.

PCR reactions (20 μl) contained 0.05 mM dNTPs, 0.2 μM of oligonucleotide primers, 1 × PCR buffer, 1.5 mM $MgCl_2$ (for GAPDH and p53 genes) or 1.25 mM $MgCl_2$ (for p21 gene) and 0.5 U *Taq*-polymerase (Eppendorf, Germany). Preliminary experiments were performed in order to determine the number of cycles that gave a linear relation between the number of cycles and the amount of PCR product (data not shown). Although each RNA was treated with DNase before RT, each cDNA was also checked for the presence of DNA with primers for intron 6 of the p53 gene (107 bp). For this purpose, primers IN6A (5′-AAG TCT GGT TTG CAA CTG GG-3′) and IN6B (5′-GAG GTC AAA TAA GCA GCA GG-3′) were used in 50 cycles of PCR. No PCR product was detected in any of the cDNAs indicating lack of DNA in the samples. Expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous internal control to standardize the results. GAPDH cDNA (600 bp) was amplified in 25 cycles (98°C, 10 minutes; 95°C, 4 minutes; 95°C, 30 seconds; 60°C, 30 seconds; 72°C, 45 + 1 seconds) using primers GAPDHA (5′-AAC

GGA TTT GGT CGT ATT GGG C-3′) and GAPDHB (5′-AGG GAT GAT GTT CTG GAG AGC C-3′). p53 cDNA (247 bp) was amplified in 28 cycles, under identical conditions as described, using primers ex4A (5′-GAT GCT GTC CGC GGA CGA TAT T-3′) and ex4B (5′-CGT GCA AGT CAC AGA CTT GGC-3′). p21 cDNA (400 bp) was amplified in 25 cycles using primers p21-S (5′-AGT CAG TTC CTT GTG GAG CCG GAG C-3′) and p21-2 (5′-ATG GTC TTC CTC TGC TGT CC-3′).

The PCR products were separated in native 8% polyacrylamide gels stained with SYBR Gold (Molecular Probes Inc.) and analyzed by densitometry. Calculations of the intensity of bands corresponding to specific genes were performed using Image Master VDS software (Pharmacia Biotech).

Immunocytochemistry

The cells were plated at a density of 5×10^4/well onto eight-well plastic slides (Nunc, USA) 24 hours before viral infection. At 24 hours after infection, the cells were washed with PBS and fixed in methanol with 3% hydrogen peroxide (Kemika, Zagreb, Croatia). Application of normal rabbit serum (1:10 in PBS) for 30 minutes at room temperature blocked nonspecific binding. Primary mouse monoclonal antibodies p53 (Ab-2 and Ab-3, Oncogene, USA) and p21 (Pharmingen, USA), in concentration of 5 μg/ml, were allowed to bind overnight at 4°C. After washing the slides in PBS, secondary antibody (rabbit anti-mouse, DAKO, Denmark) was applied for 1 hour at room temperature. Finally, the slides were stained with 0.0025% diaminobenzidine tetrahydrochloride (Sigma) containing 4% H_2O_2 for 7 minutes and counterstained with hematoxylin for 30 seconds. The slides were analyzed with a light microscope (Olympus, BH-2). The level of nonspecific background staining was established for each measurement using control cells processed in the same way but without exposure to the primary antibody.

Detection of apoptosis

Nucleosomal DNA fragmentation analysis. Apoptotic DNA fragments were isolated according to the method described by Herrmann et al.[26] Briefly, cells were plated at 10^6 in 10-ml plates and infected. At 24 hours after infection, attached and floating cells were harvested, washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 seconds with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 minutes at 1600 *g*, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 hours with RNAse A (final concentration 5 mg/ml) at 56°C followed by digestion with proteinase K (final concentration 2.5 mg/ml) for 2 hours at 37°C. After addition of .5 vol. 10 M ammonium acetate, the DNA was precipitated with 2.5 vol. ethanol, dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA), and separated by electrophoresis in 1% agarose gels.

Annexin V test. Detection and quantification of apoptosis and differentiation from necrotic cells at single-cell level was performed using Annexin-V-FLUOS staining kit (Roche, Germany), according to the manufacturer's recommendations. After a desired length of time, both floating and attached cells were collected. The cells were then washed with PBS, pelleted and resuspended in staining solution (Annexin-V-fluorescein labeling reagent and propidium iodide (PI) in Hepes buffer). The cells were then analyzed under a fluorescence microscope. Annexin-V (green fluorescent) cells were determined to be apoptotic, and Annexin-V and PI cells were determined to be necrotic. Percentage of apoptotic cells was expressed as a number of fluorescent cells in relation to nonfluorescent cells, which was expressed as 100%.

Statistical analysis

One-way ANOVA was used to test the significance of the differences between the samples using Microcal Origin, Microcal Software, Inc., USA.

Results

Effect of Ad5CMV-p53 and Ad5CMV-p21 on cell growth

We investigated the effect of exogenous wt p53 and p21 expression on the growth of cancer cell lines, MCF-7,

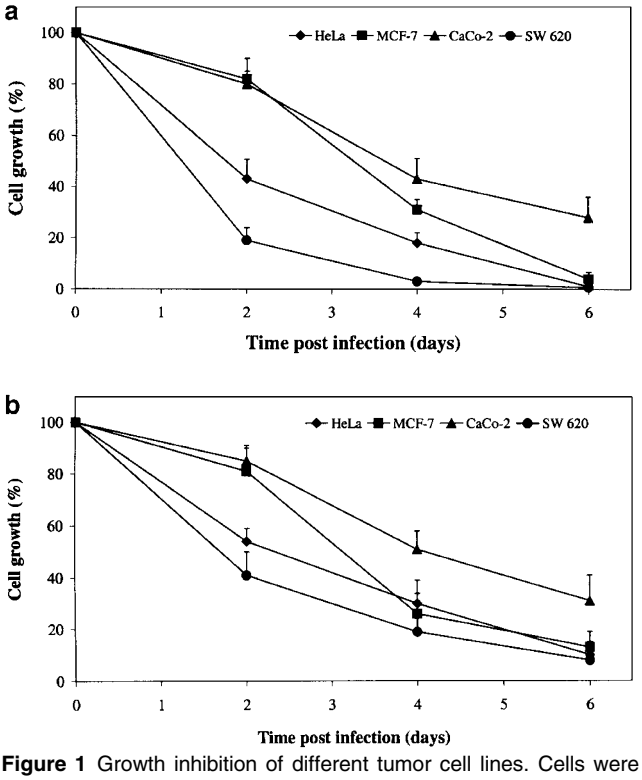

Figure 1 Growth inhibition of different tumor cell lines. Cells were plated in triplicates on 24-well plates and the growth was determined by counting the cell numbers at different time points after infection with Ad5CMV-p53 (**a**) and Ad5CMV-p21 (**b**) at a MOI 50. Results represent means ± SD. The experiment was repeated three times, and the results were smilar.

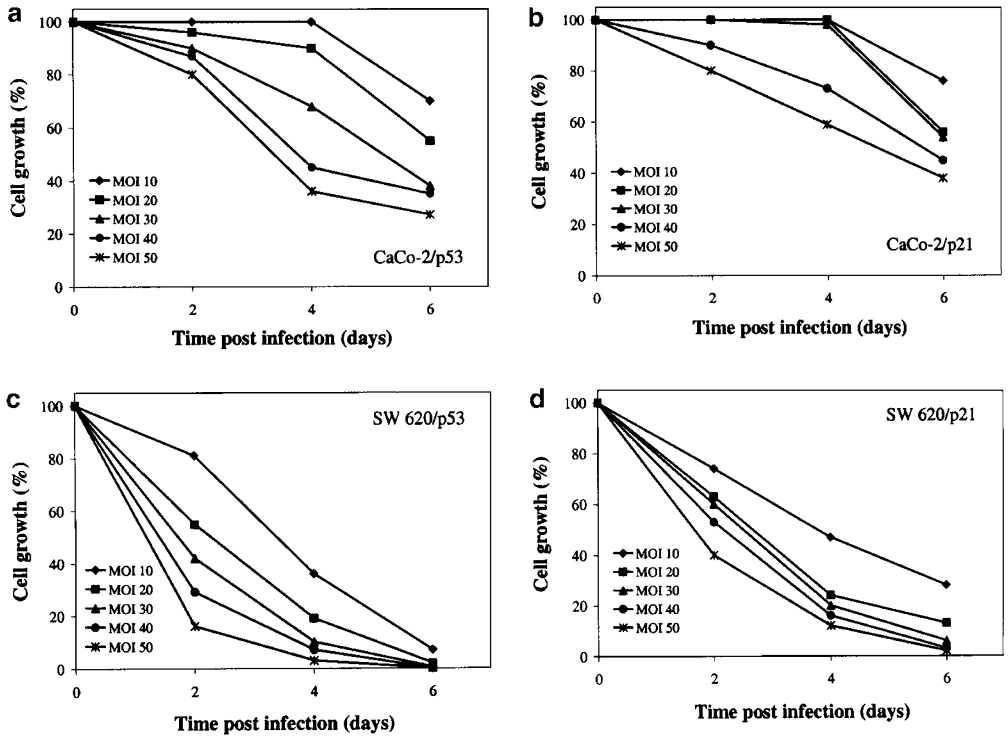

Figure 2 Time and dose response curves obtained after infection of CaCo-2 (**a** and **b**) and SW 620 (**c** and **d**) cell lines with Ad5CMV-p53 (**a** and **c**) and Ad5CMV-p21 (**b** and **d**) at different MOI values ranging from 10 to 50 PFU/cell. Cells were plated in triplicates on 24-well plates and cell number was counted at different time points. Results represent mean of one of three experiments.

HeLa, SW 620 and CaCo-2, having different origin and genotypes. Each cell line was exposed to 10–50 MOI (PFU/cell) of either Ad5CMV-p53, Ad5CMV-p21 or dl-312 (control) vector. Cells infected with the control vector had growth rates similar to control (uninfected) cells (data not shown). Adenoviral vectors Ad5CMV-p53 and Ad5CMV-p21 strongly inhibited the growth of all cell lines tested in a time- (Fig 1) and dose (Fig 2)-dependent manner. However, the inhibition varied among the cell lines tested and, the difference in the inhibitory effect was the most obvious at the second day after infection. For example, 2 days after infection with Ad5CMV-p53 at MOI 50, significant differences were found between MCF-7 and HeLa ($P = .00116$), as well as between CaCo-2 and HeLa cells ($P = .00716$). The difference between MCF-7 and CaCo-2 cells, on one side, and SW 620 cells on the other was even more significant ($P < .0001$). The growth inhibition differences between the cell lines tested were lost by day 6, except for CaCo-2. Moreover, about 40% of CaCo-2 cells survived even 6 days after infection with Ad5CMV-p53 at MOI 50, while the growth of other cells was completely inhibited over the 6-day period (Fig 1). Similar results were obtained after infection with AdCMV-p21, although it was slightly less efficient than Ad5CMV-p53, but this difference was significant only for SW 620 cells ($P < .05$). Figure 2 shows time and dose response curves of the most resistant (CaCo-2) and the most sensible (SW 620) cell lines. These results suggested that infection with both vectors had a more profound growth inhibitory effect on cell lines, with either mutant (SW 620) or inactivated (HeLa) p53 protein than on cells with wt p53 (MCF-7), except CaCo-2 cell line (mutant p53).

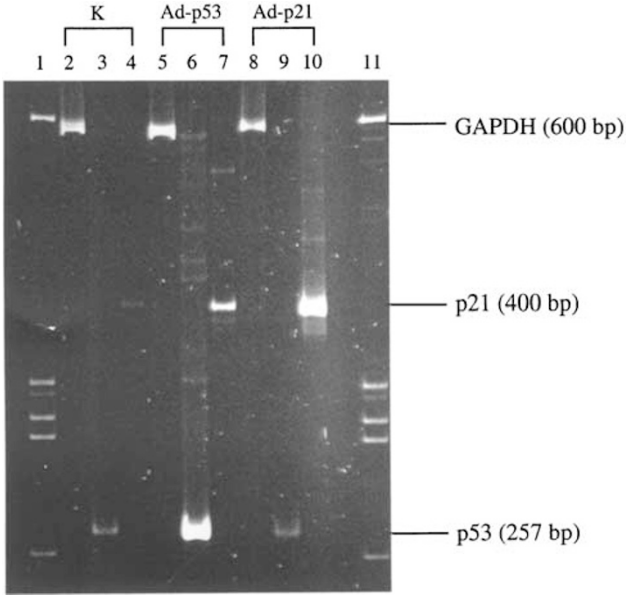

Figure 3 Semiquantitative RT-PCR analysis of p53 and p21 genes expression in HeLa cell line. RT-PCR products were resolved in 8% native polyacrylamide gel and stained with SYBR Gold. Lanes 1 and 11: DNA molecular weight marker ΦX174/Hae III; lanes 2–4: uninfected HeLa cells, lanes 5–7: HeLa cells infected with Ad5CMV-p53; lanes 8–10: HeLa cells infected with Ad5CMV-p21.

p53 and p21 gene expression following Ad5CMV-p53 and Ad5CMV-p21 infection

The RNA expression level of p53 and p21 genes, in control and infected cells, was examined 24 hours after infection with both vectors at MOI 50. As expected, results confirmed increased p53 expression after infection with Ad5CMV-p53. The level of p53 RNA increased approximately four-fold in HeLa (Fig 3), MCF-7 and SW 620 and 14-fold in CaCo-2 cells (not shown). Similarly, after infection with Ad5CMV-p21, the level of p21 RNA increased approximately five- (MCF-7) to eight-fold (HeLa), dependent on the cell line.

Moreover, RT-PCR revealed induction of p21 in all cells infected with Ad5CMV-p53. However, p21 RNA levels after infection with Ad5CMV-p21 were significantly higher (three-fold in MCF-7 and CaCo-2, and four-fold in SW 620 and HeLa) than the level achieved after p21 induction with Ad5CMV-p53. Infection with control vector (dl-312) did not cause any substantial change in the expression of p53 or p21 gene (data not shown).

p53 and p21 protein expression following Ad5CMV-p53 and Ad5CMV-p21 infection

The results confirmed that the exogenous p53 and p21 mRNA produced from cells infected with Ad5CMV-p53 and Ad5CMV-p21, respectively, are efficiently translated into proteins. Immunocytochemical analysis revealed the

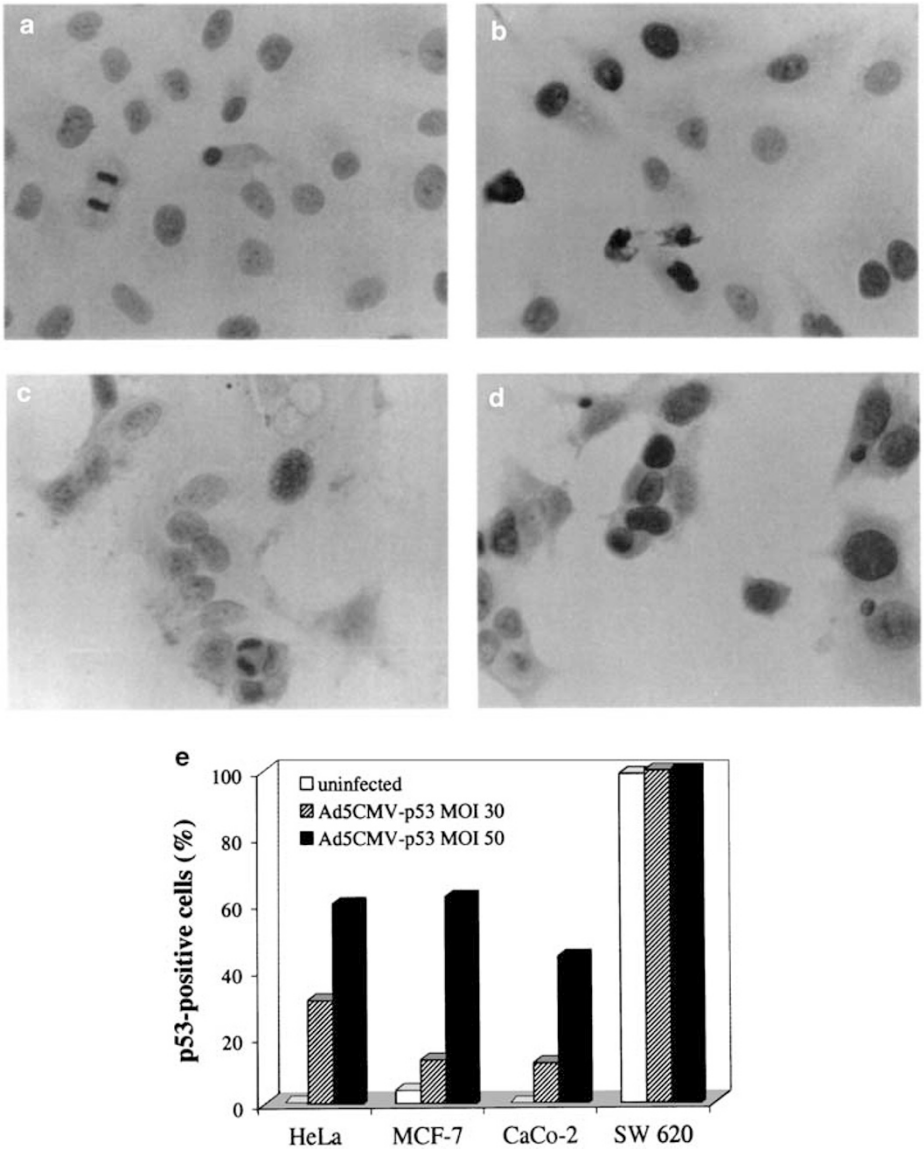

Figure 4 Immunocytochemical analysis of p53 protein expression in HeLa, MCF-7, CaCo-2 and SW 620 cell lines. The cells were infected with Ad5CMV-p53 at MOI 30 and 50 and compared to control cells. Immunocytochemical staining of HeLa (**a** and **b**) and MCF-7 (**c** and **d**) cells is shown: control cells (**a** and **c**), Ad5CMV-p53-infected cells (**b** and **d**). Percentages of p53-positive cells, obtained in one of three similar experiments, are shown in panel f.

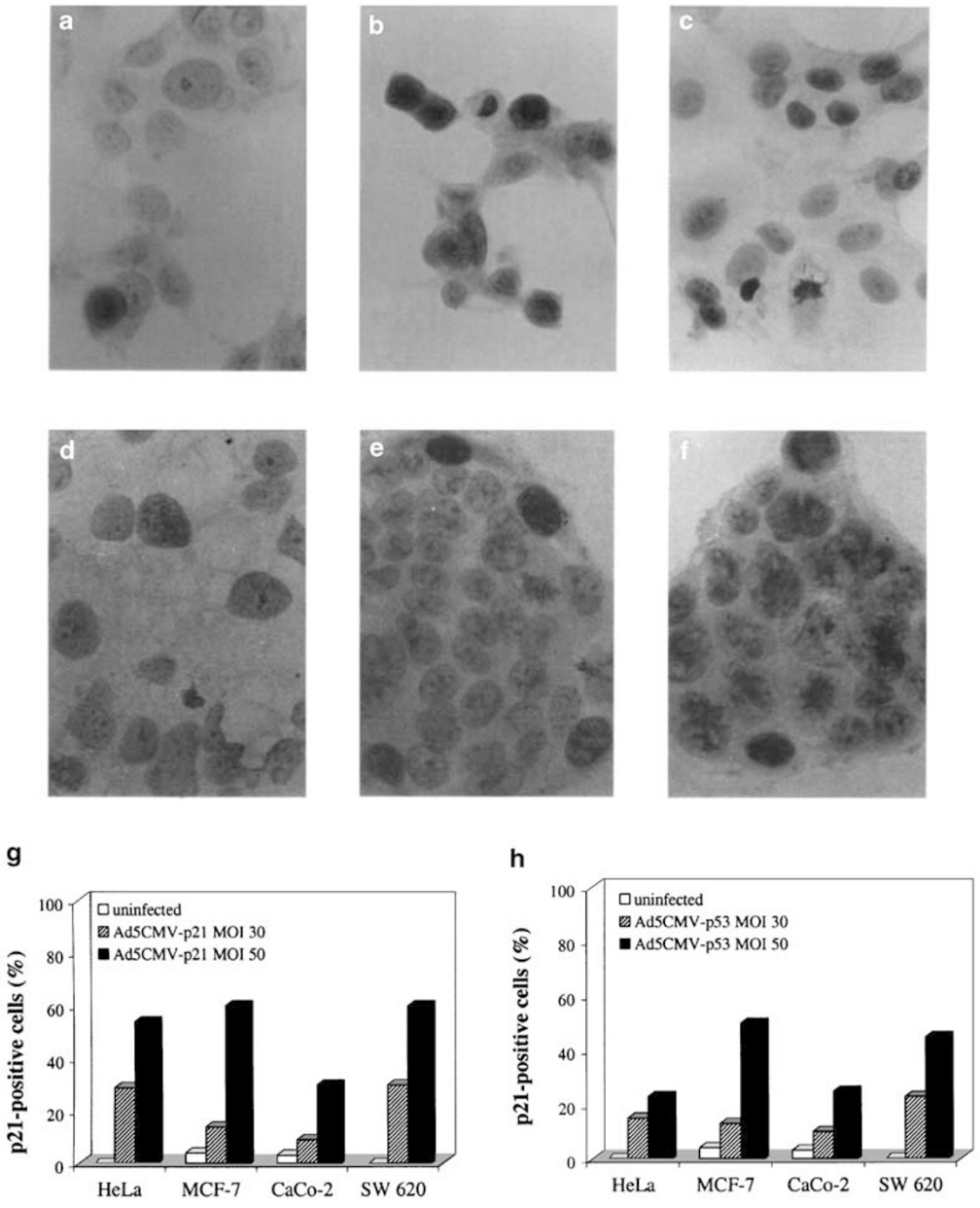

Figure 5 Immunocytochemical analysis of p21 protein expression in HeLa, MCF-7, CaCo-2 and SW 620 cell lines. The cells were infected with Ad5CMV-p53 and Ad5CMV-p21 at MOI 30 and 50 and compared to control cells. Immunocytochemical staining of MCF-7 (**a**–**c**) and CaCo-2 (**d**–**f**) cells is shown: control cells (**a** and **d**), Ad5CMV-p21-infected cells (**b** and **e**) and Ad5CMV-p53-infected cells (**c** and **f**). Percentages of p21-positive cells after infection with Ad5CMV-p21 (panel g) and Ad5CMV-p53 (panel h) are shown.

characteristic nuclear staining of both proteins (Figs 4 and 5). After infection with Ad5CMV-p53, the percentage of p53-positive cells increased with the MOI of the virus in all cell lines tested except in SW 620 (Fig 4f). An increase in p53 in this cell line was not readily discernible as control cells also express high level of the p53 protein. On the contrary, we were unable to detect any positive uninfected CaCo-2 cell and only less than 1% HeLa cells (Fig 4f).

Almost identical results were obtained by the analysis of the p21 protein after infection with Ad5CMV-p21 (Fig 5a, b, d, e, g), except in SW 620 cell line, which was negative for p21 protein. However, after infection, 30 and 60% of p21-positive cells were detected, depending on the MOI (Fig 5g). Interestingly, about 3% of uninfected CaCo-2 cells stained positively for p21.

As expected, immunocytochemistry confirmed increased p21 expression after infection with Ad5CMV-p53 in all cell lines tested (Fig 5c, f, h). However, there were less p21-positive cells and/or the brown staining was much less intensive than in Ad5CMV-p21-infected cells

(Fig 5c and f). This is in accordance with the results obtained with RT-PCR.

Effect of p53 and p21 overexpression on apoptosis

Annexin V assay was performed 24, 48 and 72 hours after viral infection at the MOI 50. The mean values from three different experiments show that 24 hours after the infection with Ad5CMV-p53, apoptosis was induced in HeLa and SW 620 cell lines (30 ± 5 and 18 ± 2% of apoptotic cells, respectively) (Fig 6a and b). This was confirmed by the detection of DNA fragmentation (Fig 7). Annexin V positivity increased during the next 48 hours in HeLa, but stayed constant in SW 620 cells. No significant increase in the number of Annexin V positivity was evidenced in MCF-7 and CaCo-2 cells infected with Ad5CMV-p53 or Ad5CMV-p21 in respect to control or dl-312-treated cells ($P > .05$). Interestingly, control CaCo-2 cells showed relatively high percentage of Annexin-V-positive cells, which varied among experiments (6–17%). At 24 hours after exposure of cells to 50 MOI of Ad5CMV-p21, a slight, but significant ($P = .02$) apoptotic response was found in SW 620 cells. This was confirmed by DNA fragmentation analysis that revealed apoptotic DNA fragments 24 hours after infection (Fig 7). Moreover, 48 and 72 hours after p21 transduction, a significant number of apoptotic cells was observed in HeLa and SW 620 cells as well (Fig 6a and b). These results indicate that p21 gene could also activate apoptosis in the cell lines with mutant or inactivated p53 protein, but after prolonged incubation with the adenoviral vector.

Discussion

In recent years, there has been increasing interest in understanding the biochemical mechanisms involved in the regulation of cell cycle progression and cell death. Numerous studies have suggested that the cell cycle is modulated by the interactions of different cell cycle molecules.[24] The critical decisions of a cell to grow, differentiate or enter quiescence, senescence or programmed cell death are controlled by a number of interconnected and complex signaling pathways. Mutations in any of the genes involved in these regulatory pathways represent a potential pathway of carcinogenesis. Therefore, the ultimate goal of basic research on cell cycle regulation and carcinogenesis is to develop new treatment strategies as alternative or adjuvant to conventional radiation and chemotherapy.

The high frequency of p53 mutation in diverse human cancers implies that the loss of p53 function is central to tumor development.[5] Consequently, much effort has been made to understand complex p53 biology and to recognize its role in diagnostic or prognostic benefit or in improving cancer therapy. Mutations of p53 affect its ability to suppress tumorigenesis mostly through the loss of wt p53 function, and also through dominant-negative effect of mutant over wt p53 or gain of additional oncogenic properties. The presence of p53 mutation has been associated with unfavorable prognosis in a variety of tumor types, disease progression and often with enhanced resistance to many antitumor agents.[1,9] Thus, the gene replacement therapy with wt p53 provides an attractive strategy to cancer treatment. Indeed, numerous studies

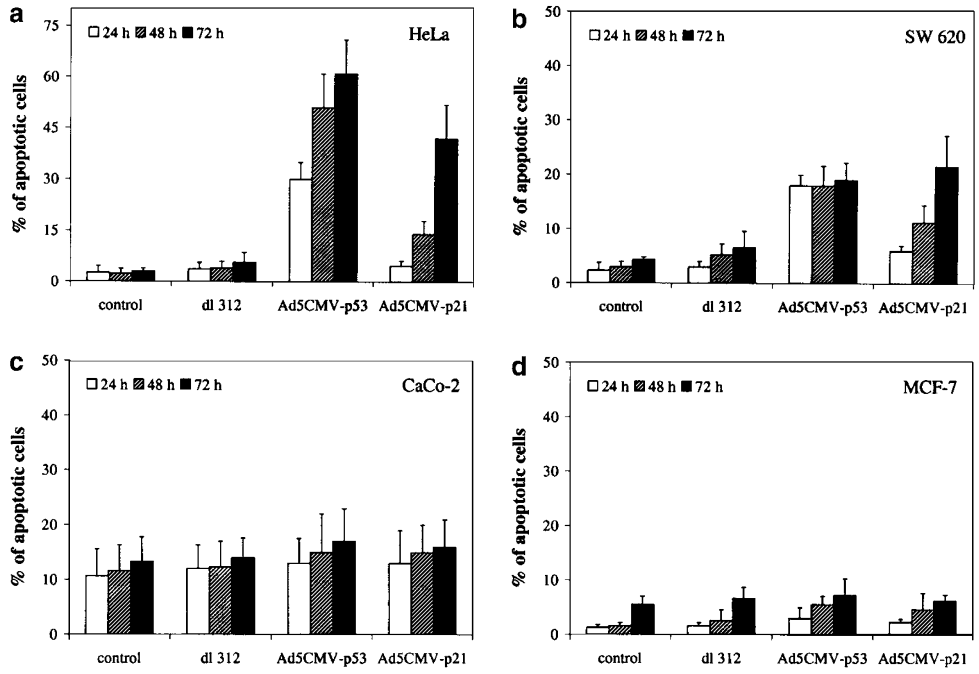

Figure 6 Percentage of apoptotic cells. The cells were either mock infected or infected with dl 312, Ad5CMV-p53 and Ad5CMV-p21 at a MOI 50 and examined at different time points using Annexin V assay. (**a**) HeLa, (**b**) SW 620, (**c**) CaCo-2 and (**d**) MCF-7 cells. Results represent means of three experiments $\pm$ SD.

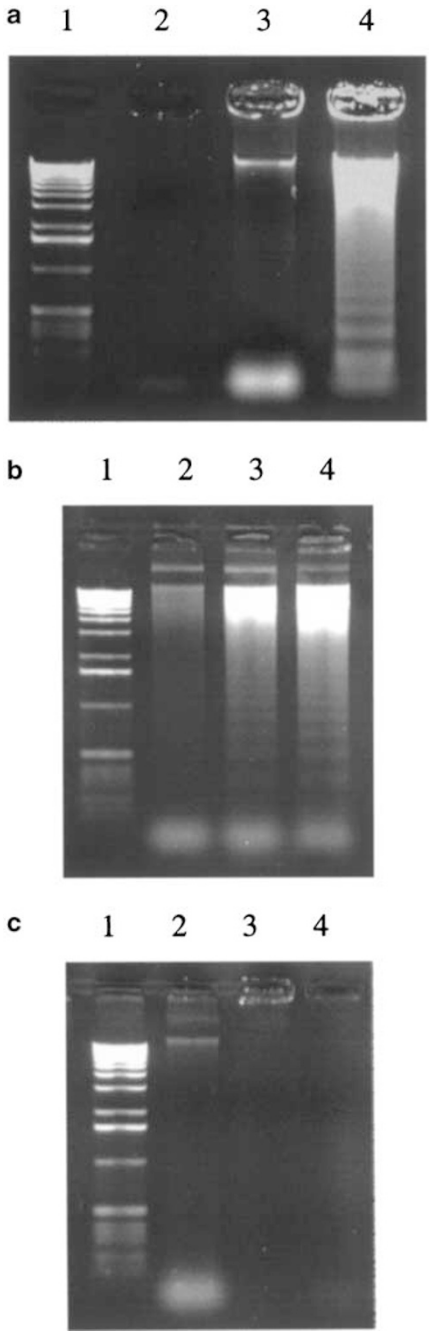

Figure 7 Nucleosomal DNA fragmentation in HeLa (**a**), SW 620 (**b**) and MCF-7 (**c**) cells. Low molecular weight DNA was isolated from attached and detached cells 24 hours after infection with Ad5CMV-p53 and Ad5CMV-p21 at a MOI 50. Lanes 1: DNA weight marker, lanes 2: DNA from control cells, lanes 3: DNA from cells infected with Ad5CMV-p21, lanes 4: DNA from cells infected with Ad5CMV-p53.

have confirmed that reintroduction of the wt p53 suppresses tumor cell growth, induces apoptosis and/or increases sensitivity to conventional antitumor agents (detailed review by Nielsen and Maneval[8]). Moreover, the response to p53 overexpression was usually correlated with the endogenous p53 status, being more pronounced in p53^{mut} or p53^{null} than in p53^{wt} or in nontransformed cells.[8,9,27] This effect is mostly attributed to apoptosis that is activated in cells, which were either p53^{mut} or p53^{null} prior to p53 transduction, although there are also different reports, as mentioned in the introduction. Among thousands of genes that play a role in the regulation of apoptosis, p53 is among the most important ones.[7] Therefore, the studies of molecular mechanisms responsible for the divergent activities of p53 gene in different cells are of great importance.

The purpose of this study was to correlate the efficacy of p53 and p21 overexpression in human tumor cell lines that differ with the endogenous (prior to infection) p53 and/or p21 status. Namely, in the cells containing functional p53 gene product, the cell growth and apoptosis are obviously regulated through different interactions between regulatory molecules than in the cells with no functional p53 protein. Furthermore, expression of p21 protein is thought lately to counteract the apoptosis, as was shown in melanoma and colorectal carcinoma.[20,28] In spite of this, the role of p21 in apoptosis still remains controversial, since it has also been reported to induce apoptosis in human breast,[14,29] endometrial[15] and cervical[30] carcinoma cells.

Our findings correlate with previous studies (regarding p53 overexpression) that demonstrate more pronounced inhibitory effects and/or induction of apoptosis in null or mutant p53 cells. The strongest growth inhibitory effect, as well as induction of apoptosis, was shown in SW 620 (mutated), and in HeLa (inactivated p53) cells after infection with Ad5CMV-p53. An exception to this rule is the CaCo-2 cell line, which was previously suspected to have a mutation in the p53 gene, because neither p53 nor p21 expression was influenced following exposure to DNA-damaging agent.[24] Indeed, Djelloul et al[23] confirmed one mutant (stop codon in position 204) and one deleted p53 allele. Regardless of the mutation, this cell line was the most resistant to either p53 or p21 overexpression. The growth of MCF-7 (wt p53) was much less affected than the growth of SW 620 and HeLa cells and apoptosis was not detected. Similar results on MCF-7 cells were obtained by Katayose et al.[16,31]

There is evidence that the functional status of pRb may be an important determinant in dictating the cellular outcome of p53 activation. For instance, pRb could inhibit or protect cells from apoptosis.[32,33] Besides, abrogation of pRb function through expression of the HPV E7 gene (as it is the case in HeLa cells) facilitates the conversion of p53-mediated growth arrest into apoptosis.[34] Moreover, Ad5-p53-induced apoptosis in HeLa cells was partially inhibited when Ad5-Rb was added simultaneously.[35] On the other hand, deletions and loss of expression of the Rb gene are rare in human colorectal carcinomas,[36] and Okamoto et al[37] showed that pRb is expressed in SW 620 cells, so the induction of apoptosis in this cells could not be the result of pRb inactivation. Therefore, we presume that the induction of apoptosis in HeLa and SW 620, and not in MCF-7 and CaCo-2 cells, may be predicted by the endogenous status of p21 gene.

Moreover, it could actually be because of the fact that p21 could protect against p53-mediated apoptosis. To be precise, the cells with either no mutant or inactivated p53 protein show little or no detectable basal levels of p53-inducible p21 protein, while the cells that express endogenous wt p53 show detectable basal levels of this protein. On the contrary, in our experiments, in CaCo-2 cells a basal level of p21 was readily detected. As it was shown by Gartel et al,[24] this cell line differentiates in culture while reaching confluence and p21 is induced in a p53-independent manner.

However, all cell lines exhibited p21 mRNA and protein induction following Ad5CMV-p53 infection. We could not detect any correlation of p21 induction levels between cell lines, in regard to p53 status, as other authors did. For instance, several authors[10,13,31] confirmed higher level of induced p21 in p53^{mut} or p53^{null} cells, while some others[20,38] showed higher level in p53^{wt} cells. In this regard, the possible protective role of p21 protein against apoptosis could be because of the endogenous (basal), not the inducible, levels of p21 protein. This is in accordance with the work of Polyak et al.[28] Accordingly, our results obtained with Ad5CMV-p21 infection showed that p21 overexpression itself induced apoptosis in the cells with mutant (SW 620) and inactivated (HeLa) p53 protein, both of which have no p21 basal levels, but after prolonged period of incubation with the virus. On the contrary, we could not detect apoptosis in MCF-7 cells, although we also noticed giant cell formation after infection with Ad5CMV-p21 (data not shown) as did Sheikh et al.[14] Besides, the growth inhibitory effect of Ad5CMV-p21 was comparable to that of Ad5CMV-p53, as it was also shown by Katayose et al.[16] Almost the same results as ours were obtained by Tsao et al[30] on HeLa cells. They showed that sense p21 adenovirus infection at a MOI 25 caused suppression of the cell growth, detachment from culture surface and confirmed apoptosis 48 and 96 hours after infection. Moreover, p21-induced apoptosis was not prevented by overexpression of the bcl-2 transgene. Similar results were also obtained by Ramondetta et al.[15] They showed the induction of apoptosis in cells infected with p21 after 72 and 120 hours and indicated that p53 was more efficient in activating apoptosis than p21. Shibata et al[29] also showed that the restoration of p21 function in mammary tumor cells can significantly reduce tumor growth *in vitro* and *in vivo* through inhibition of cell growth and increased apoptosis. Importantly, in all of the mentioned studies, the cells had inactivated, mutated p53 and/or lacked p21 expression. It seems that p21 inhibits tumor cell growth both by arresting cell cycle and inducing apoptosis. However, p21 activated slower and milder apoptotic pathway than p53, which is obviously p53-independent. On the contrary, high level of p53 expression induces apoptosis that masks the cell-cycle-arrest response.[39]

How then could such a role be explained of the same gene product and why the overexpression of p21 also induces apoptosis only in cell lines with no endogenous p21? One possible explanation could be the ratio of expression between p21 and p53, as it is shown by Gorospe et al,[20] since at low doses (prior expression) p21 protected cells from apoptosis, while at high levels (a higher MOI), p21 alone induced cell death. Another possible proof for this is shown by Duttatory et al.[40] They report that the additional increase of p21 beyond the basal level, seen in serum-deprived quiescent cells, is associated with apoptosis indicating the importance of quantitative threshold effect.

Although p21 was identified as the first cyclin-dependent kinase inhibitor and was involved in p53-dependent and p53-independent cell cycle arrest, since then many different possible interactions and networks regarding this molecule have been discovered.[21] Some of them could be attributable to its antiapoptotic function: binding of procaspase 3, interaction with caspases 8 and 10 and inhibition of apoptosis-regulating kinases, such as SAPKs and ASK1.[21,41] Moreover, although p21 is not a transcription factor, some of its functions may be mediated by indirect effects of p21 on cellular gene expression. Analysis of p21-activated genes suggested that p21-induced growth arrest may be accompanied by a paracrine growth-stimulatory effect, because several of these genes encode secreted proteins with mitogenic or antiapoptotic activity.[42] Besides, p21 was reported to bind to different transcription factors and modulate their function. For instance, it can induce expression of proteins, which have either anti- or proapoptotic function.[42] Thus, the observed induction of pro- and antiapoptotic genes by p21 may also explain the contradictory reports on its both positive and negative effects on apoptosis. Moreover, it was shown that p21 can bind to N-terminus of c-Myc, interfere with c-Myc-Max association and suppress c-Myc-dependent transcription.[43] At the same time, c-Myc binds to C-terminus of p21, in competition with PCNA and thus counteracts p21-dependent inhibition of DNA synthesis. The balance of the reciprocal inactivation between c-Myc and p21 may determine the course of cellular processes such as cell proliferation, differentiation and apoptosis.[43] Accordingly, Frederick et al[44] showed that suppression of c-Myc using antisense oligonucleotides, in the absence of p53, was sufficient to trigger apoptosis in Tu-138 cells. Thus, inactivation of c-Myc-Max association by p21 could be one of the possible mechanisms of its proapoptotic activity in the absence of functional p53, which was the case in our study.

To conclude this discussion, we would like to cite a recent review by G P Dotto:[21] 'It is the regulatory network, itself, which has to be understood, as the function of given molecules cannot be dissociated from the context in which they operate'.

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