

Influence of p53 tumor suppressor protein on bias of DNA repair and apoptotic response in human cells

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A network of interacting cellular components is known to mediate the regulatory role of tumor suppressor protein p53 in genomic stability. DNA repair machinery is considered to be one of these vital cellular components. To investigate the modulatory function of p53 on the repair of DNA damage and related effects, we have studied the responses of human p53-wild-type (p53-WT), p53-mutant (p53-Mut) and p53-nullizygous (p53-Null) cells following exposure to UV irradiation. Absence of wild-type p53 function coincided with an enhanced sensitivity to UV, as well as induction of apoptosis. However, the lack of wild-type p53 expression did not affect the response of its signal transducer protein, p21. Repair analysis of specific genomic sequences, at a single nucleotide resolution, revealed that the removal of cyclobutane pyrimidine dimers in a non-transcribed strand was significantly slower in p53-Mut and p53-Null cell lines compared with the normal p53-WT cells. However, the repair of the transcribed strand was comparable in the three cell lines. Thus, p53 is required for the efficient nucleotide excision repair (NER) of the global genomic DNA, but not for the transcription-coupled repair of the essential genes. The decreased global NER, due to the lost p53 function, seems to be responsible for the conjoined cytotoxicity and apoptosis of human cells subjected to DNA stress damage.

Introduction

Passage of a genotoxin exposed mammalian cell, through distinct steps of multistage carcinogenic pathway, involves different etiological factors, as well as varying biochemical and genetic processes (1). The carcinogenesis initiation step is triggered through the alteration of the key cellular target genes, either by the products of endogenously operating normal cellular processes, or by interaction with exogenous physical and chemical agents in our environment (2). The genomic alterations that affect expression or function of the genes controlling cellular growth and differentiation, are considered to be the main contributors in the etiology of cancer (1,3). Thus, cellular proto-oncogenes and tumor suppressor genes have been identified as the molecular targets that suffer changes

Abbreviations: CPD, cyclobutane pyrimidine dimers; LFS, Li-Fraumeni Syndrome; LMPCR, ligation-mediated PCR; MTT, methylthiozole tetrazolium; NER, nucleotide excision repair; p53-Null, p53-nullizygous protein; p53-Mut, p53-mutant protein; p53-WT, p53-wild-type protein; TCR, transcription-coupled repair.

upon interaction with mutagenic carcinogens, resulting in their activation/inactivation and onset of neoplastic process (4–6).

Living cells have evolved sophisticated DNA repair mechanisms to overcome the instability resulting from deleterious genomic base structural alterations (3,7–10). The importance of DNA repair for maintaining normal cellular integrity, function and prevention of neoplastic phenotype is clear from numerous studies. Absence or defective repair is linked to increased susceptibility of cells to toxic, mutagenic and carcinogenic effects of genotoxin exposure (11). Complex biochemical processes, which recognize and eliminate specific adducts by diverse mechanisms, enable cells to overcome the consequences of genotoxic damage (12,13). Nucleotide excision repair (NER) eliminates a broad range of lesions induced by various physico-chemical agents (13). NER along the genome is non-uniform and this heterogeneity, termed preferential or transcription-coupled repair (TCR), has been observed with different gene sequences in organisms ranging from bacteria to human cells (7,14). Several recent investigations on tumor suppressor and other select genes have begun to provide some novel and provocative insights (3,15–17). For example, signature mutation causing lesions are more likely to occur on the non-transcribed strand of the *p53* gene (18). UV-induced cyclobutane pyrimidine dimers were shown to be repaired faster in the transcribed strand of *p53*; the strand bias of repair was particularly distinct in xeroderma pigmentosum group C fibroblasts (19). Besides TCR, heterogeneity of repair of mammalian genomic sequences is apparent at several other levels. For example, in addition to slow repair observed for cyclobutane pyrimidine dimers (CPD) in upstream promoter sequences, 10-fold faster repair rates are seen at the transcription initiation sites in the human *Jun* gene (20). The fast repair near the transcription start site was also visible in the non-transcribed strand. A gradient of DNA repair, with the rate decreasing towards the 3'-end of the gene was distinguishable for the transcribed strand.

Whereas the lesion localization and repair is an important consideration, only limited studies have been performed on integrating gene-specific damage, and the nature and role of repair modulatory factors in mammalian cells. It is becoming increasingly clear that the DNA damage response is complex and occurs in concert with transcriptional induction, cell cycle arrest and DNA replication (21). Attempts to define functional links amongst these processes, have implicated various proteins that are directly and indirectly affected upon genotoxic damage. Of these, the tumor suppressor gene, *p53*, is deemed critical for maintaining genomic stability and homeostasis (22). DNA damage induces a very early and rapid increase in nuclear wild-type p53 protein level (23). It is believed that p53 activation signals the G₁ cell cycle checkpoint (24) to delay the transit from G₁ to S, thus preventing the effect of DNA damage on vital gene functions (23). If DNA repair is not successfully accomplished, p53 may promote death of affected cells by triggering apoptosis, thereby preventing the propaga-

tion of genetic defects to successive cell generations (25). The possibility that p53 plays a role in DNA repair itself, is most alluring. In support of this, p53 has been shown to bind to single-stranded DNA, and associate *in vitro* and *in vivo* with NER proteins ERCC3, XPB, XPD and CSB (26–28). The fact that p53 regulates a number of downstream genes, and that it possibly interacts physically with both damage and repair enzymes, suggests that it could have multiple effects on NER and other repair processes. A few studies, performed to address this question, have so far been equivocal. Despite binding to basal excision repair proteins, p53(-/-) mouse cells are shown to exhibit repair rates for UV photoproducts at the wild-type rates and both show similar resistance to UV (29,30). However, mutant or disrupted p53-containing cell lines are shown to exhibit compromised NER pathways for processing DNA damage (31–33). Among these, two studies have used indirect host cell reactivation assay, and conclude that p53 or p53-regulated products function in the NER pathway (32,33). Other studies seems to indicate a role in global, but no modulation of, TCR and concludes that loss of p53 may lead to greater instability by reducing the efficiency of DNA repair (31,34). Understanding the effect of p53 and p53-regulated gene products on nucleotide repair, via excision of damage in the transcribed or non-transcribed strands, remains an important area of genetic toxicology. Systematic research is bound to uncover and clearly establish critical interconnections of DNA repair with constitutive and inductive cellular processes.

This report describes the response to genotoxic UV radiation exposure in human cells having wild-type, mutant or no p53 protein. The effect of DNA damage on cellular cytotoxicity and apoptosis was determined in conjunction with the repair of global, gene-, strand- and site-specific repair. The results demonstrate p53 protein dependent and independent differential effects on the expression of cell cycle regulatory protein p21, cellular sensitivity and apoptosis. Quantitative comparisons of repair rates within cells of different p53 backgrounds have shown that the expression of wild-type protein did not substantially affect the TCR of the transcribed strand, whereas an enhancement of repair was apparent for the repair of the non-transcribed strand.

Materials and methods

Cell culture and treatment

The normal human (p53-WT) fibroblasts (OSU-2) were established in culture as described by Venkatchalam *et al.* (35). Li-Fraumeni syndrome (LFS) fibroblast strains, MDAH087 [p53-mutant (p53-Mut), harboring a codon 248 single base substitution] and MDAH041 [p53-nullizygous (p53-Null), harboring a codon 184 frameshift mutation], both >200 population doubling, post-crisis p53 homozygous cell strains, were kindly provided by Dr Michael Tainsky (M.D. Anderson Cancer Center, Houston, TX). The fibroblast cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37°C, in a humidified atmosphere of 5% CO₂. For experiments to assess DNA damage and repair, the monolayer cells were grown to confluence in 150 mm dishes and then placed with medium without serum for 24 h. The medium was removed, cells washed with prewarmed PBS, irradiated with the desired dose of UV light (254 nm) and maintained in fresh serum deficient medium for varying times post-treatment. For protein induction and apoptosis studies, the exponentially growing cells in 60 mm dishes were washed and replaced with a thin layer of PBS, and irradiated with varying UV doses. The irradiation was carried from a germicidal lamp at a dose rate of 0.5 J m⁻²/s as measured by a Kettering model 65 radiometer (Cole-Palmer Instrument Co., Vernon Hills, IL).

Cell survival assay

Cytotoxicity was evaluated using clonogenic survival and methylthiozole tetrazolium (MTT) metabolic viability assay. Exponentially growing cells were plated at appropriate densities in 100 mm dishes and exposed to varying

UV doses after overnight attachment. The cells were then cultured for about 14 days in growth medium, then washed, fixed and stained with 0.1% crystal violet. Colonies of >50 cells were counted, and the survival adjusted for plating efficiency of cell types was calculated and plotted. For MTT assay, asynchronously growing cells were transferred into 96-well tissue culture plates (Corning) in 200 µl of complete medium. Each well contained 5 × 10³ cells. After 24 h incubation, the medium was removed from the adherent cells, washed once with PBS and the columns of 8-wells irradiated with increasing doses of UV light. The medium was replaced and the incubation was continued for another 24–72 h. Cell viability in octuplicate wells was assessed by the ability of cells to convert the soluble salt of MTT into an insoluble formazan precipitate (36). Aliquots of 50 µl of MTT (1 mg/ml) were added to each well for 2 h and the color formed was quantitated by a spectrophotometric plate reader (Biotek) following solubilization in DMSO. The cell viability was expressed relative to the untreated control wells.

Quantitation of CPD by immuno-slot blot assays

The amounts of initial dimer formation and the damage remaining in DNA after cellular repair were quantitated using non-competitive immuno-slot blot assay, essentially as described earlier (35,37). Briefly, after UV exposure and desired incubation periods, cells were recovered by trypsinization and immediately lysed for DNA isolation by a salt precipitation procedure (38) modified in our laboratory. The high quality and molecular size of the DNA was determined by agarose gel electrophoresis. DNA concentration was estimated using absorption at 260 nm and the microdiphenylamine assay (39). For damage estimation at each point, several increasing concentrations of unirradiated, irradiated and repaired DNA samples were evaluated by standard immunoassay using dimer-specific polyclonal antibodies, as described earlier (40). The damage levels were calculated by comparing the band intensities of the samples with UV irradiated DNA standard samples run in parallel with all the blots.

Gene-specific repair analysis by ligation-mediated PCR

The repair of CPD at specific sites within the exon 5 transcribed and non-transcribed strand sequences of the p53 gene was determined by ligation-mediated PCR (LMPCR) according to the original principle of damage detection at single nucleotide resolution (41). DNA was specifically cleaved at CPD and then these sites identified on sequencing gels. Samples of 10 µg of purified genomic DNA isolated from cells, exposed and incubated after UV irradiation, were treated with 10 µl of 10 × T4 endonuclease buffer and 1 U T4 endonuclease V enzyme (Epicenter Technologies, Madison, WI), and incubated at 37°C for 1 h. After increasing the concentration of DTT to 10 mM, the samples were treated with 5 µg of DNA photolyase (the generous gift of Dr Aziz Sancar, University of North Carolina). The mixture was pre-incubated at room temperature and then samples were photo-reactivated with a 360 nm black light for 1 h at room temperature. After extraction with phenol–chloroform, the DNA samples were ethanol precipitated and DNA pellets dissolved in HE buffer (10 mM HEPES, pH 7.5; 1 mM EDTA). The DNA was again quantitated by microfluorimetry and a 1 µg aliquot was tested on alkaline agarose gels to determine the dimer cleavage efficiency. Another 1 µg aliquot was used for primer extension by Sequenase 2.0 (US Biochemicals, Cleveland, OH), followed by ligation of the linker and amplification by PCR. The sets of three primers for transcribed strand (TS): 5-1 = 5'-CACTTGGTGCCTGACTTTCAAC-3', 5-2 = 5'-TGCCCTGACTTTCAA-CTCTGTCTCC-3', 5-3 = 5'-CAACTCTGTCTCCTCCTCTTCTACAG-3' and non-transcribed strand (NTS), 5-4 = 5'-GAGCAATCAGTGAGGAAT-CAGA-3', 5-5 = 5'-GGAATCAGAGGCCTGGGACCCT-3', 5-6 = 5'-TGGGGACCCTGGGCAACCA-3' were used essentially according to the detailed published protocols to generate truncated fragments (42). The products were separated on an 8% urea-polyacrylamide sequencing gel, electroblotted to a nylon membrane and probed with PCR generated, ³²P-labeled single-stranded exon 5 specific probes. The filters were used to expose a phosphorimager screen, and the individual band intensities quantitated upon imaging and processing by Imagequant software (Molecular Dynamics). The filters were also used to expose Kodak X-OMAT film for autoradiography. The LMPCR analysis was done in replicate and the repair experiments repeated with two independent samples.

Protein analysis by western blotting

The cells irradiated with varying UV doses were incubated for indicated times and recovered by trypsinization. The cells were lysed by boiling for 10 min in sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 10 mM dithiothreitol in 62 mM Tris-HCl, pH 6.8, 10 µg/ml pepstatin and 10 µg/ml leupeptin). Western blot analysis was performed with aliquots from an equivalent number of cells, as described earlier (43). Protein extracts from ~1.2 × 10⁵ cells were separated in 8 or 12% SDS-polyacrylamide gels, and transferred to PVD membranes by using a semi-dry electroblotter (Hoeffer, San Francisco, CA). Equal protein loading was confirmed from visualization

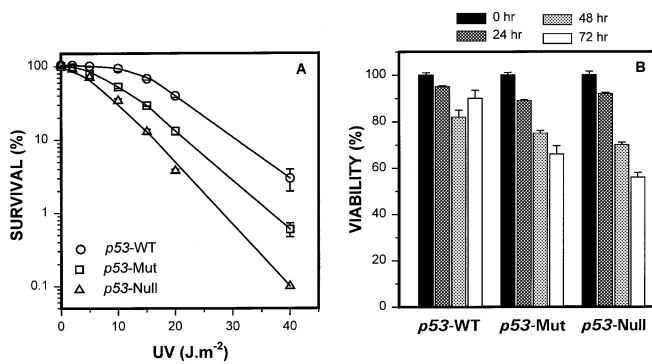


Fig. 1. Effect of p53 function on cell survival and viability to UV irradiation. Human fibroblast cells of varying p53 status were irradiated with increasing doses of UV light, and the cell survival and viability determined as described in Materials and methods. All cultures were in the exponential growth phase at the time of irradiation and were assayed at varying times after exposure. (A) UV dose-dependent cell survival scored by colony formation 14 days after irradiation. The points shown are mean \pm SE of three individual dishes. (B) Relative cell sensitivity to 20 J/m² UV at 24, 48 and 72 h post-irradiation. The points shown are mean \pm SE of eight replicate samples.

of the membranes stained for 15–20 min with fast green (0.1% fast green, 5% glacial acetic acid and 20% methanol). For p53 protein detection, a mixture of anti-p53 protein antibodies (p53 Ab-2 and p53 Ab-6 from hybridoma clones 1801 and DO-1, respectively) was used at a 1:200 dilution. The Ab-3 antibodies for the detection of p21 (clone DCS60.2) were also used at 1:200 dilution. All these antibodies were obtained from Neomarkers, Fremont, CA. The membranes were developed following treatments with goat anti-mouse IgG horseradish peroxidase conjugate (Boehringer Mannheim, Indianapolis, IN). Detection of protein specific bands was conducted through the peroxidase activity using the enhanced chemiluminescence substrate reaction (Pierce, Rockford, IL) essentially according to the manufacturer's instructions using Kodak X-OMAT AR film. Rainbow protein size markers were used in all the gels to localize the gel transfer regions for specific proteins and determine the transfer efficiency. All experiments were repeated at least three times and the results of representative experiments are shown in various figures.

DNA fragmentation analysis

DNA fragmentation analysis was performed essentially as described (39,43). Briefly, at indicated times the adhered cells were gently trypsinized, mixed with any unattached cells removed with the medium and pelleted by centrifugation. DNA from pelleted cells was recovered by a unique differential cellular lysis procedure that separates fragmented DNA released only by apoptotic cells from native intact DNA of unaffected cells by centrifugation. The 13 000 g supernatant was treated with RNAase-A for 30 min at 37°C followed by Proteinase K for 1 h at 45°C. Aliquots of DNA corresponding to $\sim 1 \times 10^6$ cells were resolved by gel (1.2% agarose) electrophoresis and visualized by ethidium bromide staining. The apoptotic index was calculated from the amounts determined for the fraction of released fragmented DNA to the total DNA in the initial cell pellet (39).

Results

Differential cell type specific UV cytotoxicity

The effect of p53 protein on the inherent cellular sensitivity was determined in three different cell lines from the loss of clonogenic survival following UV exposure. Maximum differential of cell survival was apparent in the low UV dose range. In general, the normal human fibroblasts exhibited higher resistance to UV than the fibroblasts with mutant p53 or no p53 protein (Figure 1A). The cells lacking p53 showed the greatest reproductive death by UV irradiation. These results were fully supported by the short-term viability assessment of the irradiated fibroblast cells. The cellular viability following 24, 48 and 72 h of exposure to increasing doses of 254 nm UV showed significant differences in the UV induced cytotoxicity in cells with normal or dysfunctional p53 (Figure 1B). Whereas all three cell lines showed a dose-dependent

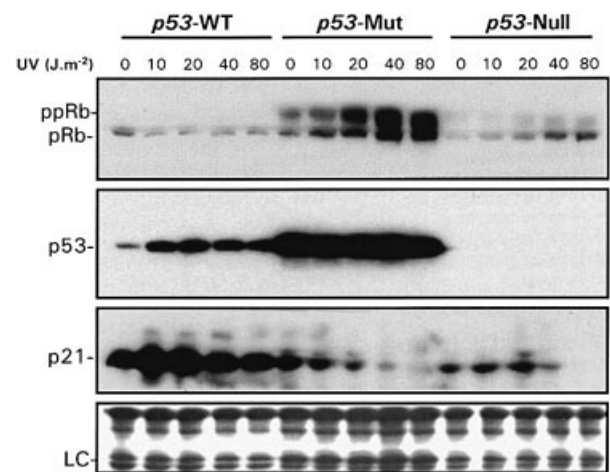


Fig. 2. UV dose-dependent responses of pRb, p53 and p21 proteins in human cells. Exponentially growing fibroblast cell types were exposed to indicated doses of UV radiation and incubated further in complete medium. At 8 h post-treatment, the proteins were released upon lysis of cells as described in the text. Cellular extracts (equivalents of 1.2×10^5 cells/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, developed with specific antibody to different proteins and visualized by enhanced chemiluminescence as described in Materials and methods. Fast green staining of portion of the protein blots was used as loading control (LC).

decrease in the extent of dye metabolizing functional cell population, the cells with normal wild-type p53 were relatively more resistant and those lacking expression of p53 were most sensitive to UV treatment. Like clonogenic survival assay, cells expressing the mutant form of p53 were found to have intermediate sensitivity. The order of sensitivity response was identical, whether cell viability was compared at 24, 48 or 72 h after irradiation. However, the extent of observed cytotoxic effect was most pronounced when cells were allowed a 72 h period to express or recover from the radiation induced damage. The viability of cells showed a marked post-incubation time decrease for p53-Mut and p53-Null cells, but a significant recovery in the viable p53-WT cells at 48 and 72 h of incubation.

Regulatory protein induction responses

Normal human fibroblasts, containing wild-type p53 allele, and the corresponding cells with altered p53 protein, showed distinct induction responses for various regulatory proteins upon genotoxic UV treatment (Figure 2). No detectable change in wild-type protein levels was seen in p53-WT cells maintained in normal culture conditions for 24 h. However, exposure to doses as low as 2 J/m² showed an increase in p53-WT protein specific signal, compared with that of normally maintained constitutive levels in the cells. Earlier time course experiments indicated that the rapid induction response, detectable within a few hours and peaking at about 6–8 h, was sustained up to a period of at least 24 h (43). A several-fold increase in the induction of p53 was seen with incremental doses of UV seemed to peak at ~ 10 J/m². Higher doses showed only slight signal enhancement. The p53-WT cells also showed p21 protein levels consistent with the presence of p53-WT protein. However, p21 protein levels increased only slightly in cells for 8 h after treatment with increased UV doses. Quantitative assessment revealed that, at higher doses of genotoxin, the p21 protein levels decreased as a function of the dose. Contrary to responses of the normal cells, the homozygous p53-Mut

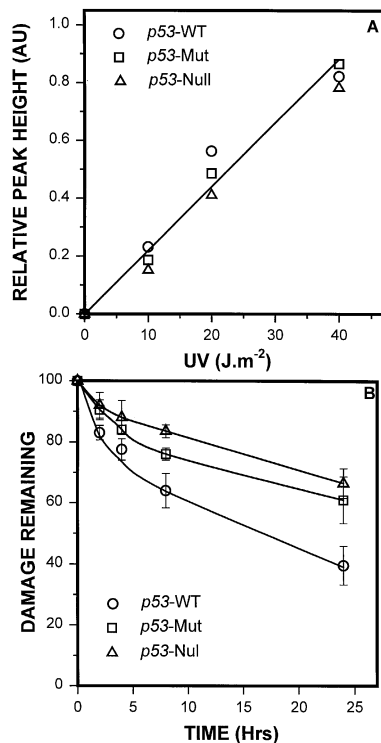


Fig. 3. Induction of DNA damage and repair in human fibroblast cells by UV irradiation. (A) Dose-dependent induction of CPD. The cells in monolayers were irradiated with varying doses of UV radiation and immediately lysed for DNA isolation. The extent of CPD formation was immunologically determined by the antibody-binding sites, as described in Materials and methods. Each point is an average of three independent determinations obtained from identical amounts of NC filter bound DNA. For clarity, standard deviation of mean values (<5%) is not shown in the plot. (B) Global NER of UV induced CPD. The repair was determined as the loss of antibody binding sites from DNA obtained from indicated cell types at varying times after irradiation with a UV dose of 20 J/m². For each point, four to six different DNA concentrations were used and the extent of DNA damage was determined from the linear portion of the curve. The amount of damage was calculated upon comparison with a standard reference and the repair expressed as the percentage of initial damage remaining at the indicated time.

cells indicated constitutively high levels of the mutant protein, owing to the inherent stability of this protein form. No discernible change in the p53 protein levels was apparent upon DNA damage of cells by increased doses of UV. The p53-Mut cells had relatively lower levels of p21 protein that decreased with the increasing doses of UV. As expected, no detectable p53 protein could be observed in a two-fold excess protein extract of p53-Null cell line. Surprisingly, these cells also exhibited a clear expression of p21 protein that could be sustained at low UV doses, but like the other two cell lines decreased at doses in excess of 40 J/m².

Global genomic DNA repair

Relative extent of DNA damage by UV and the repair of CPD were determined immunologically in the human fibroblast cells by damage specific antibodies in a sensitive slot-blot assay. The antibodies used in these experiments were previously characterized to specifically recognize CPD and binding of the antibodies was abolished upon enzymatic photoreactivation of the antigenic dimer sites (37,40). Cell types of varying p53 functional status were irradiated with 20 J/m², and the levels of CPD measured immediately or subsequent to their repair in cognate cells. The CPD formation was dependent upon the

dose of UV irradiation (Figure 3A). Although much lower amounts of damage from low dose exposures could be immunologically detected, the dose response results shown were obtained with a lower amount (100 ng) of immobilized DNA to ensure a linear and quantifiable antibody binding response. The three cell lines did not seem to exhibit a discernible difference in the formation of CPD in their genomic DNA by the increasing UV exposure up to 40 J/m². Thus, a dose of 20 J/m² was chosen for repair studies. The overall amount of CPD repaired and the rate of removal of CPD was highest for the p53-WT fibroblast cells. On the contrary, both the p53-Mut and p53-Null cells exhibited relatively decreased rates of NER repair and overall loss of CPD from the genome (Figure 3B). A quantitative comparison of CPD removal showed that p53-WT, p53-Mut and p53-Null cells, respectively, repaired 56, 44 and 37% of initial CPD within 24 h following irradiation. A similar repair pattern for three cell lines was also apparent at the earlier post-treatment times.

Site- and strand-specific DNA repair

To determine the repair of CPD within individual strands of specific genes, the dimer formation was mapped in select exonic region sequences of p53 gene by LMPCR. The relative induction of dimers showed expected variations of amounts according to the dimer type. Although subtle sequence context-dependent variations were seen, the frequency of dimer formation was generally highest at 5'T-T followed by 5'T-C dinucleotides and to a very small extent at 5'C-C and 5'C-T dinucleotides (results not shown). This order was consistent with previous observations of dimer induction by UVC irradiation of DNA. In addition, higher dimer frequency was clearly discernible on the 5' side of a track of adjacent pyrimidine bases. Figure 4 shows the examples of dimer specific sequence bands within selected sites of exon 5 and the loss of these bands due to repair in the three different cell types. A clear, rapid and complete loss of specific signal could be seen for both TT and TC dimers in the transcribed as well as non-transcribed strands in p53-WT cells (Figure 4A). However, the extent of this decrease was different between the transcribed and non-transcribed strands of p53-Mut and p53-Null cells. The loss of site specific signal from the transcribed strand in these cells was comparable to the transcribed strand of the p53-WT cells. However, decreased dimer removal from the non-transcribed strand in both these cells was apparent from the presence of the prominent signals at 24 as well as 48 h sample lanes. The p53 protein function-based differential removal was also visible in dimer sites within the p53 intronic sequences. For the purpose of direct comparison, the relative extent of repair in transcribed and non-transcribed strands is plotted for the same type of dimer (Figure 4B). The plots indicate that the relative repair within p53-WT, p53-Mut and p53-Null cells was similar for TC dimers residing within the transcribed strand. Greater than 50% of initial damage was repaired within 4–8 h from this strand. A similar rate of repair was observed for TC dimer sites of the non-transcribed strand in p53-WT cells. However, the rate as well as the extent of repair of these TC dimers was clearly and reproducibly lower in both p53-Mut and p53-Null cells. In these cells, 50% repair of TC dimers of the non-transcribed strand required in excess of 48 h post-treatment incubation time. This pattern of repair, i.e. low CPD repair in the non-transcribed strand within cells of dysfunctional p53 protein, was seen with most of the other potential dimer sites in p53 exons 5 and 8 (data not shown).

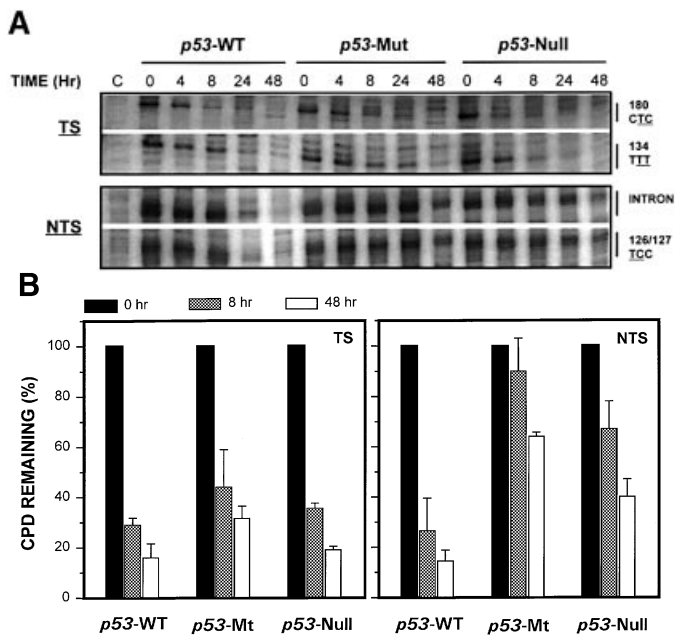


Fig. 4. Repair within different strands of *p53* gene at a single nucleotide resolution. Human fibroblast cells were irradiated with a UV dose of 20 J/m^2 and the DNA isolated at indicated post-treatment times. The repair within the transcribed and non-transcribed strands of exon 5 was determined by LMPCR as described in Materials and methods. (A) Representative autoradiographs of segments of the sequencing gel, for both transcribed and non-transcribed strands, are shown for band intensity comparisons at CPD sequences in p53-WT, -Mut and -Null expressing cells. (B) Repair profiles of select CPD sites in DNA of cells of different p53 status are shown for the transcribed and non-transcribed strands of *p53* gene. Bands corresponding to different individual CPD sites were quantitated by phosphorimager analysis. The percentage of repair was determined upon comparison with the intensity from bands at 0 h. Any background due to non-specific cleavage at the potential dimer sites seen in control unirradiated DNA, lane C, was subtracted from each point. The specific CPD sites were localized from Maxam-Gilbert sequencing lanes run alongside each LMPCR assay.

Cellular apoptosis

The relationship of cellular sensitivity and apoptosis resulting from genotoxic exposure of the human fibroblast cell lines was ascertained by subjecting the UV irradiated cells to DNA fragmentation analysis. The cells were irradiated with increasing doses of UV radiation and evaluated for DNA fragmentation at several times post-treatment. All the cell types exhibited a UV dose-dependent increase in the amount of fragmented DNA released upon incubation for periods of 24–48 h (results not shown). For direct comparison of the extent of apoptotic DNA fragmentation, a UV dose of 20 J/m^2 was chosen for the incubation time-dependent increases of the cellular DNA fragmentation (Figure 5). Negligible amounts of fragmented DNA was seen in p53-WT cells immediately after irradiation. A small and gradual increase in the released DNA was seen as the time of incubation of cells was prolonged. However, gel electrophoresis of DNA showed that, even after 48 h of UV exposure, the liberated DNA from p53-WT cells retained the molecular size in excess of 50 kb (Figure 5A). On the contrary, both p53-Mut and p53-Null cells demonstrated substantial release of fragmented DNA during the post-treatment incubation. Furthermore, the size of the DNA fragments from these cells was greatly reduced, reaching the expected 180 bp nucleosome monomer size for the p53-Null cells at 48 h incubation time.

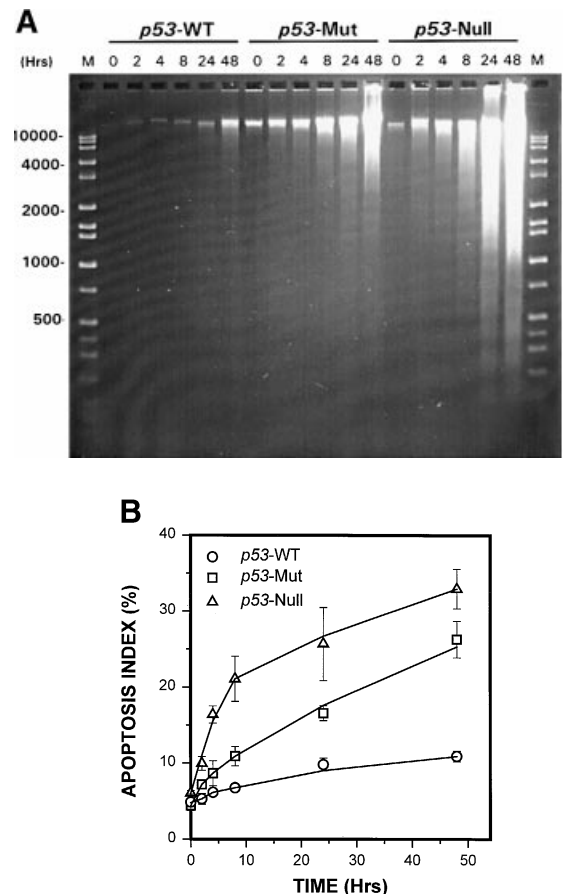


Fig. 5. Effect of UV irradiation on the induction of apoptosis. Fibroblast cell types of varying p53 status were irradiated with a UV dose of 20 J/m^2 and maintained under full growth medium for various periods. The cells were recovered at indicated times and evaluated for apoptosis as described in Materials and methods. (A) Fragmented DNA from apoptotic cells was separated and analysed by agarose gel electrophoresis and ethidium staining. Lanes marked M are Hi-Lo DNA size markers. (B) The extent of apoptosis was quantitatively determined by measuring the fraction of cells releasing the fragmented DNA. The data shown represent at least three independent experiments.

Upon quantitative assessment of apoptotic index of irradiated cell populations, the overall extent of fragmentation was also shown to be low, intermediate and highest for p53-WT, p53-Mut and p53-Null cells, respectively (Figure 5B). All three cell lines showed a basal level of ~5% cells that failed to retain the DNA upon differential lysis. This may represent an assay related background or a true fragile cell fraction in these lines. This percentage notwithstanding, the amount of the cells undergoing apoptosis increased only slightly, up to 10% in the case of p53-WT cells. This was followed by an increase in apoptotic index so that, with p53-Mut cells, it gradually reached ~28% at 48 h. Once again, the highest level of apoptosis was seen with p53-Null cells that not only showed an increased overall extent, but also a rapid induction of this response following irradiation with 20 J/m^2 . As expected, the quantitative values of apoptotic index fully complemented the results of gel electrophoretic analysis. Flow cytometric analysis of the three cell lines also showed a clear UV dose-dependent increase in the sub-G1 apoptotic cells in irradiated p53-Mut and p53-Null cells, and an absence of such cells in p53-WT fibroblasts (data not shown).

Discussion

Several direct and indirect observations with various genotoxins have shown that the fate of DNA damage at a given location, and the consequent biological effect, is strongly influenced by many factors. In general, it is indicated that (i) the cell survival correlates with the efficient repair of essential genes rather than overall repair capacity, (ii) the repair kinetics vary with the functional status of damaged sequences, (iii) the actively transcribing strand of a gene is repaired at a more rapid rate than its non-transcribing complement, (iv) the lesion adjacent sequence determines the target repair efficiency, and (v) host of other known and unknown factors seem to ensure high fidelity repair of key genomic sites. Since DNA repair pathways are no longer considered in isolation of cell biology and physiology, studies are beginning to address the interconnections between vital processes involved in the maintenance of genomic stability and normal cellular functionality. The impact of one of the key effector molecules, tumor suppressor protein p53, known to possess pleiotropic properties, is being evaluated at multiple levels of biochemical interaction in living organisms. The results described here, have utilized the human cells of distinctly different p53 status and attempt to address some important interrelated questions related to the consequences of genotoxic damage.

Many studies have evaluated the possible role of p53 protein on cell survival using diverse test systems and genotoxic agents. The results, so far, have not clearly defined the mechanism by which p53 could be effecting the survivability of the exposed cells. The resulting debate could be attributed to the contradictory data emerging from the differences in experimental approaches (30). Apart from using cells derived from transgenic mice and human cells from Li-Fraumeni patients, the alteration of p53 functionality has also been achieved by selective expression of viral oncoproteins. Whereas both chemical and physical agents have been tested, a discussion restricted to studies of UV induced damage is clearly appropriate to the presented data. Amongst these, most notably the demonstration of increased clonogenic survival and cell viability of UV irradiated LFS fibroblast cells is coincident with loss of wild-type p53 function (31). This kind of cytotoxic response seems to fit very well with the accepted dogma, that wild-type p53 ensures the elimination of genetically non-restorable and critically damaged cells. Yet this crucial observation is not supported by other similar studies when the UV cytotoxicity is compared among cells with functional or compromised p53 function (33). Data presented in Figure 1 also show greater UV sensitivity in LFS fibroblast cells having either a mutant or lost p53 function. Whereas p53-WT cells also exhibit a UV dose-dependent decrease in clonogenic survival and viability, these cells are relatively resistant and able to promptly recover under full growth conditions. The cells with dysfunctional p53 are unable to show such recovery and do not produce a higher fraction of clonogenic cells in a survival assay, as observed with the same LFS patient fibroblast cells used in a study by Ford and Hanawalt (31). Due to the contradictory nature of these results, we further tested the cells for the expression of p53 and other regulatory proteins, and data clearly show a UV radiation induced response consistent with the expected cellular phenotype (Figure 2). Excess proteins failed to show a detectable amount of p53 protein in the p53-Null cells and the altered protein form was fully maintained by p53-Mut cells at elevated constitutive levels, irrespective of the level of damage by increased UV doses.

The p53 protein modulation of biological consequences of genotoxic damage has been explained by its potential role in damage repair and several studies suggest a direct participation of this protein in the damage excision process. Different systems and approaches have also been used to assess the effect of p53-WT on the efficiency of repairing genotoxic DNA damage. Indirect repair assessment, by determining the UV modified plasmid DNA reactivation in cells with normal and abrogated p53 seem to indicate a role for the tumor suppressor protein (33). Reduced repair in the absence of p53-WT was also revealed upon direct measurement of repair of UV induced lesions in LFS fibroblast of inherently different p53 status (31,34). In addition, the latest studies have helped to further differentiate between the human cellular repair types that seem to be affected by p53 protein. For example, a decreased rate of repair was only seen at the level of global repair that excises DNA damage, irrespective of the functional status of the genomic DNA segments being repaired (34). The gene specific TCR is unaffected and appears to operate with equal efficiency, irrespective of the nature of p53 function in human cells. The results presented here are in agreement with these studies and show a significant difference in the NER rates of human LFS fibroblasts. These results were also obtained by direct quantitation of induced dimers, using the well-established dimer specific antibodies. Under conditions where dose-dependent initial damage levels were identical, the p53-WT expressing cells were more efficient in dimer elimination than either of the cells containing mutant or no p53 protein. Further in-depth analyses of repair at the level of individual strands have revealed dramatic and possibly more meaningful differences in the repair rates. It is well known that the heterogeneity of DNA repair of actively transcribing gene sequences can be attributed to the targeting of repair complex to the transcribed strand of the essential genes. Accordingly, the rate and extent of repair of the non-transcribed strand occurs at a relatively lower quantitative level than that of the transcribed strand. In this process, an absence of p53-WT function has been shown to further exacerbate this differential in normal repair proficient cells (34). Thus, it is inferred that p53 may not be a necessary component of the active TCR complex, but might be involved in global repair that is also responsible for the lesion elimination within the non-transcribed strand. A close comparison of the presented data for global NER and TCR at various sites of individual strands also identified the diminished repair for the non-transcribed strand of *p53* gene sequence. So p53 protein appears to directly contribute to what can be visualized as the deficiency in global NER. In this regard, the data presented, using a powerful site specific repair assay, is comparable with the other results of repair rates averaged over the entire transcribed and non-transcribed strands of the *p53* gene (31,34). These undisputed observations, however, do not explain the basis of this specific deficiency promoted by the lack of a functional p53 within apparently repair-normal cells. Because the inability of efficient repair of the non-transcribed strand is clearly implicated in both mutation and carcinogenesis, there is a definite need to discern the role of p53 in manifestation of these biological consequences. Based on the existing knowledge regarding the regulatory function of p53 and the many different gene products affected by its function, it is indeed possible to speculate for a direct and indirect mechanism by which p53 protein may also regulate DNA repair. Any of the relevant cross-acting products like p21, GADD45, PCNA,

XPB, XPC, XPD and XPE can be envisioned to have a potential involvement (11). Thus, direct experimental studies, aimed at addressing the mechanistic aspects of modulating NER by p53 protein, would reveal the definitive nature of critical association between the vital cellular gene products.

The decreased genomic repair of cells lacking p53-WT function should predispose them to decreased relative survival to UV exposure. However, presence of p53-WT function has also been shown to enable the exposed cells to arrest at G1/S stage of cell cycle and, in several cell types, prompt apoptotic cell death. This is generally a cell type dependent response and human fibroblast cells are known to exhibit a resistance to undergo apoptosis following a genotoxic insult (22,43,44). The presented data are in agreement with a clear lack of apoptosis, despite expression of p53-WT function, in normal human fibroblast cells. On the contrary, both the cell types, either bearing a mutant p53 or no p53 protein, readily undergo apoptosis following UV irradiation. Interestingly, the degree of apoptosis index was highest for p53-Null, intermediate for p53-Mut, and least or negligible for the p53-WT cells. This trend paralleled the global genomic repair, as well as the repair of the non-transcribed strand, but not the TCR observed in these cells. The apoptotic response was apparent within 4–8 h of UV irradiation. At later times, the universal hallmark of apoptosis, i.e. the oligonucleosomal DNA fragments, can be easily seen in p53-Null cells. However, it appears that UV induced damage invokes phase-I fragmentation resulting in an early release of high molecular weight DNA in both the cell types (43,45). It is obvious that apoptosis occurred by a p53-independent mechanism and gradual DNA fragmentation is causal to active cell death also scored as the loss of clonogenicity in cell survival assay. It could be argued that the presence of p53-WT protein confers UV resistance through some DNA repair related mechanism. Moreover, based on the repair data, the UV resistance can be attributed to the effective global NER that is modulated by the status of p53 protein. Our apoptosis results do not coincide with the results of similar experiments using the same cells by Ford and Hanawalt (31). That study indicated that the p53 homozygous mutant cells had ~20% apoptotic cells compared with >90% in heterozygous mutant LFS cells. This high degree of apoptosis visualized 48 h after irradiation of these cells was suggested to be due to p53-WT protein function of the wild-type allele. However, apoptosis results with normal cell-bearing p53-WT were not presented in this study. Conceivably, the homozygous p53-WT cells ought to have exhibited the highest degree of apoptosis. Instead, our data show no such response, even at relatively higher doses of UV radiation. Based on the overall data, we conclude that the apoptosis in cells lacking p53-WT is a p53-independent phenomena, but dependent on UV damage as the trigger. The degree of the apoptotic response depends more on the extent of initial damage and the residual unrepaired damage during early periods after exposure. Since the transcribed strand is promptly repaired irrespective of p53 protein status, it is tempting to suggest that the persistent damage within the non-transcribed strand acts as the primary trigger for the induction of apoptosis and cytotoxicity.

It is widely believed that p53 protein exerts some of its cellular regulatory effects through the kinase inhibitor p21 protein. Our data indicate that wild-type p53 is not necessary for the expression of p21 because induction response of this protein was qualitatively comparable in the three cell lines. Also, the mere presence of p21 protein is not sufficient to

evoke the apoptotic response in exposed cells. This was clear from the results of p53-WT cells that exhibit higher levels of p21 protein without significant DNA fragmentation. Interestingly, a recent study has demonstrated that 254 nm UV exposure resulted in the concomitant induction of p21 protein, as well as G1 arrest in normal human skin fibroblast and the p53-deficient fibroblast derived from cancer prone LFS patients (46). Therefore, cell cycle arrest, as well as apoptosis, believed to be mediated primarily via wild-type p53 function, can occur through a p53-independent pathway. Whether p21 itself has a role in NER has generated some conflicting reports. Two studies seem to indicate that, despite inhibition of PCNA mediated DNA replication, p21 had no effect on NER (47,48). One report suggested an inhibitory effect (49). All these experiments have relied on the *in vitro* evaluations of p21 activity in the excision repair process. Using an indirect tetracycline inducible system *in vivo*, a recent study has suggested that p21 may modulate NER to facilitate the repair of UV damage (50). Our data, while exhibiting a relatively higher repair in presence of greater cellular levels of the p21 protein, are not necessarily conclusive in implicating p21 in excision repair.

In summary, our results demonstrate a role for p53 protein in the DNA repair process and establish that the cell death can be a p53-independent response, but dependent on DNA damage of the genome that eludes repair. The mechanism of action of the p53 and p53-regulated gene products on nucleotide repair via excision of damage in the genome overall, the transcribed or non-transcribed strands, and the related biological responses remain to be fully delineated. A systematic and co-ordinated approach, using (i) human cells, (ii) defined regulatory protein background, (iii) relevant gene targets, and (iv) *in vivo* damage analysis, particularly at nucleotide resolution, should yield definitive insights to unambiguously resolve the modulating role of the critical cellular regulatory proteins on processing of DNA damage.

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