Inverse correlation between p53 protein levels and DNA repair efficiency in human fibroblast strains treated with 4-nitroquinoline 1-oxide: evidence that lesions other than DNA strand breaks trigger the p53 response

Razmik Mirzayans1,3, Saber Bashir4, David Murray1 and Malcolm C. Paterson2
1 Department of Oncology, University of Alberta, Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada and 2 Department of Biological and Medical Research (MBC-03), King Faisal Specialist Hospital and Research Centre, PO Box 3354, Riyadh 11211, Saudi Arabia

Introduction

The tumor suppressor protein p53 is a key factor in maintaining genomic stability and cellular homeostasis. This protein regulates the expression of genes, such as p21WAF1, GADD45 and BAX, that contain p53 recognition sites in their promoters (1–3). The products of these genes participate in critical cellular processes, including DNA repair (4,5), cell cycle control (6), replicative senescence (7,8) and apoptotic cell death (9). In normal human cells the half-life of the wild-type p53 protein is of the order of 5–20 min, consistent with its rapid turnover. Several studies have shown that genotoxic stress leads to stabilization and transient accumulation of p53 protein as well as potentiation of its transactivation property.

Induction of p53 by DNA-damaging agents is known to be mediated through at least two distinct signal transduction pathways. One of these pathways involves ATM, the product of the gene mutated in patients with the cancer-predisposing disorder ataxia telangiectasia (AT). This pathway is activated upon exposure to ionizing radiation and chemical compounds that induce DNA strand breaks (10). Despite intensive study, it is still uncertain whether the signal for activation of p53 by these agents resides in the nucleus (e.g. DNA strand breaks; 10–12) or other intracellular components (13,14). The second pathway for induction of p53 does not involve ATM and functions upon exposure to agents such as UV light (15–17) and cisplatin (18), which produce bulky DNA lesions. It is generally assumed that strand breaks accumulating in DNA in the course of excision repair events may be the signal for activation of p53 by UV light. Recently, however, cells belonging to complementation groups A and G of xeroderma pigmentosum (XP), a UV-hypersensitivity human disease (19), were reported to be extremely susceptible to induction of p53 following UV exposure (16,17). Since XP group A and G cells have a severe deficiency in the incision step of the ‘nucleotide’ mode of excision repair that is responsible for removal of bulky DNA lesions (19), these recent observations suggested that it is bulky DNA damage per se, and probably not incision-associated strand breaks, that triggers p53 stabilization in UV-irradiated cells (16,17).

Herein, we compare the induction of p53 in human dermal fibroblast strains from normal, AT and XP donors following treatment with 4-nitroquinoline 1-oxide (4NQO), an extensively studied model environmental carcinogen whose DNA-damaging properties encompass those of both UV and ionizing radiation (20). 4NQO requires metabolic activation to be converted to the ultimate carcinogen 4-acetoxyaminoquinoline 1-oxide (21). This metabolite reacts with DNA to form three purine adducts: 3-(deoxyguanosine-N2-yl)-4-aminopurine 1-oxide, N-(deoxyguanosine-C8-yl)-4-aminooquinoline 1-oxide and 3-(deoxyadenosine-N6-yl)-4-aminooquinoline 1-oxide (for details see ref. 22). Like UV photolyses, 4NQO–DNA adducts are removed by the nucleotide excision repair pathway in normal human cells. Although there is a significant overlap in the repair pathways operating on UV- and 4NQO-induced DNA adducts, the repair efficiency of the various fibroblast cultures to induce p53

We observed a consistent, inverse correlation between the incidence of DNA strand breaks and levels of p53 protein; >90% of strand breaks and alkali-labile sites were repaired over 2 h following treatment with 1 µM 4NQO, whereas ~3 h of post-treatment incubation was required to demonstrate a significant rise in p53 protein. In normal fibroblasts, there was no temporal relationship between the incidence of DNA strand breaks and levels of p53 protein; >90% of strand breaks and alkali-labile sites were repaired over 2 h following treatment with 1 µM 4NQO, whereas ~3 h of post-treatment incubation was required to demonstrate a significant rise in p53 protein. In contrast, exposure of normal fibroblasts to γ-rays resulted in a rapid up-regulation of p53 and the level peaked at 2 h post-irradiation. XP cells with a severe deficiency in the nucleotide excision repair pathway showed abnormally high levels of p53 protein in response to 4NQO treatment, indicating that lesions other than incision-associated DNA strand breaks trigger p53 up-regulation. We observed a consistent, inverse correlation between the ability of the various fibroblast cultures to induce p53 following 4NQO treatment and their DNA repair efficiencies. Treatment with 0.12 µM 4NQO, for example, caused a >2-fold up-regulation of p53 in excision repair-deficient (AT, XPA and XPG) strains without eliciting any effect on p53 levels in repair-proficient (normal and XPE) strains. We conclude that up-regulation of p53 by 4NQO is mediated solely by an ATM-independent mechanism and that the p53 response is primarily triggered by persistent alkali-labile 4NQO–DNA adducts.

Abbreviations: AT, ataxia telangiectasia; dThd, thymidine; 4NQO, 4-nitroquinoline 1-oxide; XP, xeroderma pigmentosum.
lesions (20), differences have also been reported. Cells belonging to XP complementation group E, for example, exhibit normal levels of repair after 4NQO treatment, but defective repair in response to UV light (23,24). On the other hand, cells from AT patients show normal repair of UV photoproducts but defective repair of 4NQO–DNA adducts (20,25). In addition to bulky DNA lesions, 4NQO generates substantial amounts of active oxygen species in the cell, thereby producing DNA single-strand breaks (but not double-strand breaks) and alkali-labile sites (26–28).

The goal of the studies reported here was to determine whether the induction of p53 by 4NQO is predominantly mediated through the ATM-dependent (ionizing radiation-responsive) or an ATM-independent (UV-responsive) pathway.

Materials and methods

Cells and culture conditions

Pertinent characteristics of the primary human fibroblast strains employed here are given in Table I. All strains were free of mycoplasma contamination and were used between passages 12 and 20. Cultures were maintained as described in Materials and methods.

Western blotting

Near confluent fibroblast cultures were seeded in 100 mm dishes (~5 x 10⁵ cells/dish) and incubated overnight. Cultures were then centrifuged and resuspended in lysis buffer (62 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue). Cellular proteins (30 µg) were separated by 10% SDS–PAGE and transferred to Hybond-P membranes (Amersham Corp., Oakville, Ontario). The membranes were washed for 1 h in blocking buffer (5% non-fat dry Carnation milk in phosphate-buffered saline) and incubated for 1 h in the same buffer containing a p53-specific monoclonal antibody (1801; Oncogene Science, Manhasset, NY). Antibody reactions were visualized by the Amersham chemiluminescence procedure using a horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary antibody (Amersham) and quantified with a digitized image analyzer (30).

Measurement of DNA strand breaks

Cellular DNA was prelabeled by incubating exponentially growing cultures in thymidine (dThd)-free medium containing 180 Bq/ml [methyl-¹³C]dThd (stock sp. act. 2 x 10⁷ Bq/mmol) for 16 h. Prelabeled cells were seeded in 60 mm dishes (10⁷ cells/dish) and incubated overnight. Cultures were then treated with 4NQO and incubated in growth medium as indicated. Velocity sedimentation of the DNA in alkaline sucrose gradients and measurements of DNA single-strand breaks were performed as described previously (30).

RNA synthesis assay

Fibroblast cultures were inoculated at 10⁵ cells/dish in 60 mm dishes and incubated overnight in growth medium (lacking dThd) and for an additional 18–20 h in medium containing 180 Bq/ml [methyl-¹³C]dThd (stock sp. act. 2 x 10⁷ Bq/mmol). After removal of the radioactive medium, cultures were treated with 4NQO (or sham-treated), incubated in fresh medium for various times and pulse labeled with 7.2 x 10⁹ Bq/ml [¹H]uridine (sp. act. 1.37 x 10⁹ Bq/mmol) for 1 h. Cells in each dish were then lyzed in a 2% SDS solution and the amount of trichloroacetic acid-insoluble radioactivity in each lysate was determined as described (31). The level of RNA synthesis was expressed as a percentage of the resultant ¹H/¹³C ratios for 4NQO-treated compared with sham-treated control cultures.

Results

Lack of correlation between levels of DNA strand breaks and p53 protein in 4NQO-treated normal fibroblasts

Normal human fibroblasts (strain GM38) were assayed for both DNA strand breakage frequency and p53 protein levels at different times after treatment with 1 µM 4NQO. As shown in Figure 1A, normal fibroblasts removed >90% of strand breaks and alkali-labile sites during the ~2 h post-treatment incubation. Significant up-regulation of p53, on the other hand, was not observed until the 3 h time point (i.e. 1 h after repair of the majority of strand breaks) and the level of p53 peaked at 6 h after 4NQO treatment (Figure 1B). For comparison, we also performed a similar experiment with cultures exposed to 10 Gy γ-radiation. Consistent with previous reports (15,32,33), radiation-induced up-regulation of p53 occurred rapidly and its level reached a maximum at 2 h post-irradiation (Figure 1B).

Abnormal induction of p53 by 4NQO in repair-deficient XP and AT fibroblast strains

Assuming that DNA strand breaks induced by ionizing radiation trigger the p53 response (11), the differential kinetics of p53 up-regulation seen in normal cells treated with 4NQO and ionizing radiation (Figure 1B) suggest that lesions other than strand breaks are primarily responsible for induction of p53 by 4NQO. To provide further support for this notion, we determined p53 protein levels in cultures of the various repair-deficient (AT, XPA and XPG) and repair-proficient (normal and XPE) fibroblast strains after treatment with 4NQO. We reasoned that if DNA strand breaks induced directly by 4NQO and strand breaks arising in the course of nucleotide excision

Table I. Characteristics of human fibroblast strains studied and their responses to 4NQO

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Clinical description</th>
<th>Sensitivity to 4NQO cytotoxicity</th>
<th>Repair of alkali-stable 4NQO-DNA lesions</th>
<th>p53 up-regulation following 4NQO</th>
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Induction of p53 by 4NQO in human fibroblasts

repair trigger the up-regulation of p53, then AT cells (which fail to up-regulate p53 in response to DNA strand breaking agents; 13,15,34) and XP cells (which do not execute the incision reaction in the nucleotide excision repair pathway; 19) should be impaired in the ability to up-regulate p53 after treatment with 4NQO.

The results of western blot analysis performed at 3 h after exposure to different concentrations of 4NQO are presented in Figure 2. The drug induced, to varying extents, up-regulation of p53 in all normal, XP and AT strains examined. In repair-proficient (normal and XPE) strains, however, a significant up-regulation of p53 was observed only upon exposure to high concentrations (>0.5 µM) of 4NQO. Compared with normal controls, the AT strains proved to be markedly sensitive to the drug, exhibiting >2-fold induction of p53 protein upon exposure to as low as 0.12 µM. Like AT strains, the XPA and XPG strains also showed relatively high levels of p53 protein in response to 4NQO.

It should be noted that certain AT fibroblast strains, including AT2BE and AT4BI used here, exhibit an abnormally increased capacity to convert 4NQO to a DNA-reactive metabolite (35). Consequently, treatment with a particular concentration of 4NQO leads to the induction of ~1.5 times higher amounts of DNA damage in AT cultures than in normal controls (Figure 3; see also ref. 35). This rather marginal difference between AT and control strains for induction of DNA damage can only partially account for the striking differences in their abilities to up-regulate p53 upon exposure to the compound (Figure 2). Unlike AT strains, all three XP strains examined sustain normal levels of DNA damage after 4NQO exposure (Figure 3).

AT fibroblasts exhibit normal recovery of RNA synthesis after 4NQO treatment

DNA photoproducts induced by 254 nm UV light are repaired much faster in transcriptionally active genes than in the genome overall (36) and there is evidence that blockage of RNA polymerase by these lesions signals the activation of p53 (16,37). Thus, cultured cells from XP and Cockayne’s syndrome patients which are defective in transcription-coupled repair show impaired recovery of RNA synthesis and extremely high levels of p53 protein following UV irradiation (16,37). These findings raised the possibility that AT cells may remove 4NQO adducts in transcribed genes at a slower rate than normal cells and this may account for the increased sensitivity of the former cells to 4NQO induction of p53. To test this possibility, we compared the rates of RNA synthesis in representative normal (GM38), AT (AT2BE) and XPG (XP2BI) strains at varying times after exposure to 4NQO. The results of two sets of experiments are presented in Figure 4. Three observations should be noted. First, despite the finding that...
4NQO at 0.12 µM induced high levels of p53 in AT and XPG fibroblasts (Figure 2), such treatment did not produce any significant effect on RNA synthesis in these strains. Second, following treatment with 0.5 µM 4NQO, normal and AT fibroblast cultures exhibited essentially similar rates of RNA synthesis at all time points examined, indicating efficient recovery of RNA synthesis in AT cells. Third, XPG cultures did not show recovery of RNA synthesis in response to a high concentration (0.5 µM) of 4NQO, which is consistent with their severe deficiency in the repair of bulky DNA lesions.

**Discussion**

It is generally held that DNA strand breaks induced by genotoxic agents signal the induction of p53 through a mechanism involving ATM (10–12). If this model is universally applicable, then cells homozygous for ATM mutations would be defective in p53 induction following treatment with 4NQO, since this treatment results in the production of DNA strand breaks both directly, mediated by free radicals (26–28), and indirectly, during excision repair of bulky DNA adducts (25,28,38). Contrary to this expectation, in the present study we have demonstrated that, compared with normal controls, cultures of the two AT strains that we examined show a markedly increased ability to up-regulate p53 following 4NQO exposure (Figure 2). These same AT strains are known to be impaired in excision repair of a particular class of alkali-stable 4NQO–DNA lesions (25). AT strains repair 4NQO-induced DNA single-strand breaks and alkali-labile sites at a normal rate; 25). In addition, we have shown here that strains representing XP groups A and G, which are characterized by a deficiency in performing the incision step in the nucleotide excision repair pathway (19,28), are also hypersensitive to the induction of p53 by 4NQO (Figure 2). On the other hand, XP group E cells respond normally to 4NQO in terms of both p53 induction (Figure 2) and DNA repair (23,24). Collectively, these results strongly suggest that persistent 4NQO–DNA adducts trigger the p53 response through a mechanism which does not involve ATM.

In this work, we report that although normal human fibroblasts remove the majority of DNA strand breaks and alkali-labile lesions within 1 h after treatment with 4NQO (Figure 1A), there is a 2 h lag before a significant rise in p53 protein level is seen and this level reaches a maximum at ~6 h post-treatment (Figure 1B). In contrast, exposure of normal fibroblasts to γ-radiation leads to a rapid up-regulation of p53 and this level peaks at 2 h post-irradiation (Figure 1B). Moreover, the AT fibroblast strains AT2BE and AT4BI, which do not up-regulate p53 over extended times (up to 48 h) following exposure to ionizing radiation (34), a DNA strand-breaking agent, are nonetheless more sensitive than normal cells to 4NQO induction of p53 (Figure 2). Taken together, these results suggest that 4NQO lesions other than DNA strand breaks and alkali-labile sites trigger the up-regulation of p53. The results of the present study also suggest that DNA strand breaks arising in the course of excision repair of bulky lesions are not a major contributor to p53 up-regulation because XPA and XPG cells, which do not accumulate such ‘incision’ breaks (19,28), exhibit a striking up-regulation of p53 in response to 4NQO exposure (Figure 2).

Our findings with 4NQO-treated cultures are, therefore, consistent with recent observations reported by us (17) and others (16,37) demonstrating that the induction of p53 by 254 nm UV light is evoked by persistent, alkali-stable DNA lesions and not by incision-associated DNA strand breaks. It has been proposed that blockage of RNA polymerase by bulky DNA lesions may trigger the induction of p53 by UV light. Evidence for this has been provided in part by the finding that XP complementation group C cells, which execute preferential repair of transcribed genes normally but are defective in overall genome repair, exhibit normal induction of p53 in response to UV exposure, whereas cells from patients with Cockayne’s syndrome, characterized by a specific deficiency in transcription-coupled repair, show abnormal sensitivity to induction of p53 by UV (16,37). In the present study, however, 4NQO at a concentration (0.12 µM) that produced little, if any, effect on RNA synthesis (Figure 4A) triggered high levels of p53 protein in repair-deficient AT and XP strains (Figure 2). In addition, AT fibroblast cultures exhibited normal recovery of RNA synthesis following 4NQO exposure (Figure 4B) while exhibiting markedly increased susceptibility to 4NQO in terms of p53 induction (Figure 2). It thus appears that an alteration(s) other than blockage of RNA polymerase may be primarily responsible for triggering the up-regulation of p53 in repair-deficient human cells treated with low concentrations of 4NQO.

As summarized in Table I, the findings of the present study, in concert with those reported earlier, demonstrate a consistent relationship between the ability of human (normal, AT and XP) fibroblast cultures to up-regulate p53 after 4NQO exposure and their sensitivity to the cytotoxic effect of the compound.

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**Fig. 4.** RNA synthesis after 4NQO treatment in the indicated fibroblast strains. Cells were either treated with different concentrations of 4NQO for 30 min and incubated in growth medium for 1.5 h (A) or they were treated with 0.5 µM 4NQO (30 min) and incubated in growth medium for various times (B). The treated and control (sham-treated) cultures were pulse labeled with [3H]uridine during the last hour of each incubation period. The means (± SE) of three determinations are presented.

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These results conform with a growing body of evidence indicating a pivotal role for the p53 signaling pathway in the cytotoxicity of genotoxic agents. Activation of the p53 pathway following genotoxic stress was initially proposed as a mechanism for diminishing the cytotoxicity of these agents by activating a transient cell cycle checkpoint at the G1/S border to allow time for repair prior to the onset of DNA replication (39). Contrary to this prediction, however, p53-deficient cells lacking the ability to activate the G1/S checkpoint were shown to be less sensitive than p53-proficient cells to the cytotoxic effects of ionizing radiation (31,40–42) and 254 nm UV light (17,43). Recent studies indicate that the primary role of p53 and certain p53-regulated genes (e.g. BAX and p21WAF1) in maintaining genetic stability following DNA damage is through elimination of injured cells from the reproductively viable population, either by apoptosis or permanent growth arrest resembling senescence. Both the p53 and BAX proteins are known to be directly involved in apoptotic signaling (3,44), whereas p21WAF1 protein activates the senescence-like program (7,8,17). Anomalies in these processes are, therefore, considered to underlie the increased resistance of p53-deficient cells to the cytotoxic action of DNA-damaging agents (17,43).

In view of these findings, we conclude that hypersensitivity of AT, XPA and XPG strains to killing by 4NQO (Table I) may be associated with increased activity of the p53 signaling pathway, leading to enhanced p53-mediated apoptosis and/or p21WAF1-mediated senescence-like growth arrest. It is interesting to note that, in contrast to the observation made following 4NQO treatment, the AT fibroblast strains used here fail to up-regulate p53 protein for extended times after exposure to ionizing radiation (34). It thus appears that anomalies in different mechanisms underlie the increased sensitivity of AT strains to killing by ionizing radiation and 4NQO.

In conclusion, we have shown that the induction of p53 by 4NQO is almost exclusively mediated through an ATM-independent mechanism in human cells and that this response is primarily triggered by persistent alkali-stable 4NQO adducts.

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References


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