p53-dependent global nucleotide excision repair of cisplatin-induced intrastrand cross links in human cells

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Cisplatin is an extremely effective chemotherapeutic agent used for the treatment of testicular and other solid tumours. It induces a variety of structural modifications in DNA, the most abundant being the GpG- and ApG-1,2-intrastrand cross links formed between adjacent purine bases. These cross links account for ~90% of cisplatin-induced DNA damage and are thought to be responsible for the cytotoxic activity of the drug. In human cells, the nucleotide excision repair (NER) process removes the intrastrand cross links from the genome, the efficiency of which is likely to be an important determinant of cisplatin cytotoxicity. We have investigated whether the p53 tumour suppressor status affects global NER of cisplatin-induced intrastrand cross links in human cells. We have used a 32P-postlabelling method to monitor the removal of GpG- and ApG-intrastrand cross links from two human cell models (the 041TR system, in which p53 is regulated by a tetracycline-inducible promoter, together with WI38 fibroblasts and the SV40-transformed derivative VA13) that each differ in p53 status. We demonstrate that the absence of functional p53 leads to persistence of both cisplatin-induced intrastrand cross links in the genome, suggesting that p53 regulates NER of these DNA lesions. This observation extends the role of p53 in NER beyond enhancing the removal of environmentally induced DNA lesions to include those of clinical origin. Given the frequency of p53 mutations in human tumours, these results may have implications for the use of cisplatin in cancer chemotherapy.

Introduction

Cisplatin is one of the most successful and widely used chemotherapeutic agents. Discovered in the late 1960s, it revolutionized the treatment of testicular cancer in the late 1970s. Previously a disease with poor prognosis, the treatment of testicular cancer with cisplatin-based chemotherapy has become a paradigm for cancer chemotherapy. In total, 90–95% of patients with early stage testicular cancer are now cured, with good prognosis also for patients with metastatic disease (reviewed in ref. 1).

Cisplatin acts by forming a variety of adducts with DNA that hinder normal cellular processes and ultimately result in cell death. Of the adducts induced, the most abundant are the 1,2-intrastrand cross links formed between adjacent purine bases on the same strand of DNA. These cross links account for ~90% of the total DNA damage induced by cisplatin and are thought to be a major contributing factor to the cytotoxic effects of the drug (2,3). The intrastrand cross links cause significant bending and unwinding of the DNA double helix, ultimately resulting in the disruption of a variety of essential cellular processes. Thus, the cross links halt progression through the cell cycle, block transcription and attract a variety of proteins such as transcription factors to sequester them from their normal, essential cellular functions (4,5). Proteins involved in the mismatch repair pathway in humans also bind to cisplatin-induced intrastrand cross links, initiating futile cycles of mismatch repair and the introduction of toxic double-strand breaks (6). All these mechanisms are thought to coordinate in order to elicit the cytotoxic effects of cisplatin in tumour cells.

Cisplatin-induced cross links are recognized and removed from the genome by nucleotide excision repair (NER) (7,8). The removal of cross links from the genome of tumour cells, and consequent reduction in the frequency of the downstream cytotoxic pathways initiated in the presence of cross links, would clearly reduce the sensitivity of those cells to cisplatin. Thus, the efficiency of NER in tumour cells is likely to be an important factor in the success of cisplatin therapy. Previous work has established that the efficiency of NER of certain types of DNA damage is regulated by the p53 tumour suppressor protein. However, unlike the well-characterized role for p53 in apoptosis and regulation of the cell cycle (9,10), the precise role of p53 in DNA repair remains to be fully elucidated, in part, due to the wide variety of DNA damage induced in human cells and the number of mechanisms involved in their removal from the genome. For instance, it is known that p53 is required for the efficient global removal from the genome of UV-induced cyclobutane pyrimidine dimers (CPDs) (11–14) and adducts formed by diol-epoxide metabolites of the potent chemical carcinogens benzo[a]pyrene and benzo[g]chrysene (15–17). Global NER of UV-induced 6–4 photoproducts is, however, much less dependent upon p53 (12,13), and it is not required for transcription-coupled repair of CPDs (11–13).

Given that the p53 gene is mutated in >50% of human tumours, it is important to establish whether p53 status affects the ability of cells to remove the cytotoxic lesions induced by chemotherapeutic drugs such as cisplatin. Differences in the efficiency of NER between p53-proficient and p53-deficient cells would suggest that therapeutic outcome of DNA damage-based chemotherapy is influenced in some way by the tumour’s p53 status. Host cell reactivation analysis of cisplatin-treated plasmids has indicated that p53 affects the ability of cells to repair cisplatin-induced DNA damage (18), although this type of analysis does not distinguish between global and transcription-coupled NER, nor the different types of DNA damage induced by cisplatin. In the present study, we have sought to

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determine whether p53 regulates the repair of the cytotoxic cisplatin-induced 1,2-intrastrand cross links. We have modified existing 32P-postlabelling protocols, developed for the analysis of in vitro-induced cisplatin cross links, to determine the requirement of p53 for the removal of the major GpG and ApG cross links from the genome in human cells. The results demonstrate that p53 is required for the efficient removal of both cross links in human cells and that these cytotoxic cross links persist in p53-deficient cells. This may have implications for the use of DNA-damaging chemotherapeutic agents such as cisplatin in the treatment of tumours with differing p53 status.

Materials and methods

Materials

Antibodies used in western blotting procedures were from DakoCytomation (Denmark), Santa Cruz Biotechnologies (Santa Cruz, CA, USA) or Sigma Chemical Co. (Poole, Dorset, UK), as indicated below. Tissue culture additives and Proteinase K were obtained from Invitrogen/GIBCO (Paisley, UK). Cisplatin and all other reagents and enzymes were from Sigma Chemical Co. (Poole), except where stated.

Cell culture

041TR cells were grown as monolayers in DMEM supplemented with 10% foetal bovine serum (Bio Whitaker Europe, Belgium) at 37°C in a humidified atmosphere of 5% CO2. These cells, originally obtained from Dr G. Stark (Cleveland Clinic Foundation, Cleveland, OH, USA), were derived from spontaneously immortalized Li–Fraumeni syndrome skin fibroblasts and stably transfected with a tetracycline-regulated system for the expression of wild-type p53 (19). They were grown in the continuous presence of G418 (600 µg/ml) and hygromycin (50 µg/ml) to maintain selection pressure for transfected cells. Tetracycline (2 µg/ml) was added when suppression of wild-type p53 was required. WI88 human Caucasian foetal lung fibroblast and isogenic SV40-transformed VA13 cells, both obtained from European Collection of Cell Cultures (90020107 and 85062512, respectively), were grown as monolayers in DMEM supplemented with 10% foetal bovine serum and non-essential amino acids at 37°C in a humidified atmosphere of 5% CO2.

To induce DNA damage, cisplatin was diluted from a 2 mM stock in 0.9% sodium chloride (pH 7.4) and hygromycin (50 µg/ml) to maintain selection pressure for transfected cells. Tetracycline (2 µg/ml) was added when suppression of wild-type p53 was required. WI88 human Caucasian foetal lung fibroblast and isogenic SV40-transformed VA13 cells, both obtained from European Collection of Cell Cultures (90020107 and 85062512, respectively), were grown as monolayers in DMEM supplemented with 10% foetal bovine serum and non-essential amino acids at 37°C in a humidified atmosphere of 5% CO2.

p53 Levels in whole-cell extracts

In order to confirm that 041TR, WI38 and VA13 cells were suitable for the analysis of the role of p53 in the repair of cisplatin-induced DNA damage, western blotting was used to monitor p53 levels in whole extracts at several time points after cisplatin treatment. In order to evaluate p53-dependent transcriptional activity, levels of the downstream transactivation target p21 were also analysed, while β-actin levels were measured as a loading and transfer control. 041TR cells, homozygous p53 mutants stably transfected with a tetracycline-regulated system for the expression of wild-type p53 (19), were grown in tetracycline (2 µg/ml) when suppression of wild-type p53 was required. In the absence of tetracycline, 041TR cells showed increasing p53 levels up to 24 h after treatment with 20 µM cisplatin, with p21 levels also being expressed at high levels after 24 h (Figure 1A). 041TR cells grown in the presence of tetracycline and treated with cisplatin showed no detectable p53 or p21 at any time after treatment (Figure 1A), confirming that tetracycline suppressed the expression of transcriptionally active p53 in 041TR cells. WI38 cells treated with 20 µM cisplatin showed increasing levels of both p53 and p21 up to 24 h after treatment (Figure 1B). VA13 cells, SV40-transformed derivatives of WI38 in which p53 function is abrogated, exhibited constitutively high levels of p53 up to 24 h after treatment, but very low and almost undetectable levels of p21 (Figure 1B); this is consistent with the loss of p53 function due to its binding to the SV40
large T-antigen and inhibition of p53-dependent transcription (23–25). These experiments confirmed that 041TR, WI38 and VA13 cells were suitable models to investigate the role of p53 in the removal of cisplatin-induced DNA damage.

**32P-postlabelling analysis of cisplatin-induced intrastrand cross links**

Analysis of DNA isolated from cisplatin-treated cells revealed the presence of several radioactive products (Figure 2A). The results from this one-dimensional TLC analysis were consistent with those obtained by Blommaert and Saris (21), with two products observed in DNA samples isolated from cisplatin-treated cells that were not present in untreated control samples beyond background levels. These products exhibited an intermediate chromatographic mobility and were located between unidentified radioactive spots on the TLC plate that were also present in control samples. In order to improve separation and quantitation of the products of interest and confirm that these products corresponded with the GpG- and ApG-intrastrand cross links, 32P-postlabelled DNA samples were subjected to further analysis by HPLC. 32P-labelled material, which was excised from the spots identified after TLC and autoradiography, was subjected to high-resolution HPLC analysis, alongside 32P-labelled GpG and ApG dinucleotides. The combination of one-dimensional TLC and high-resolution HPLC confirmed that the radioactive spots identified by TLC analysis, and the peaks obtained on HPLC profiles at retention times of ~5.0 and 11.5 min, correspond with the major cisplatin-induced GpG- and ApG-intrastrand cross links (Figure 2B). These radiolabelled products were not found in control samples containing the labelling mix and deionized water. This 32P-postlabelling method was used subsequently in the DNA repair time-course experiments to measure the formation and repair of cisplatin-induced cross links.

**Formation and repair of cisplatin-induced intrastrand cross-linked adducts**

041TR cells were grown to confluence, either in the presence of tetracycline or in its absence, and then treated with 100 μM cisplatin for 2 h as described in Materials and Methods.

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**Fig. 1.** Western blot analysis of p53, p21 and β-actin protein levels in whole-cell extracts from (A) 041TR fibroblasts grown in the presence or absence of tetracycline and (B) WI38 primary fibroblasts and their isogenic SV40-transformed derivative cell line VA13. Cells were cultured and treated with 20 μM cisplatin for 2 h as described in Materials and Methods. Extracts were prepared at the times indicated following exposure to cisplatin.

**Fig. 2.** 32P-Postlabelling analysis of cisplatin-induced intrastrand cross links. (A) 32P-postlabelled samples were applied to the origin of a TLC plate, developed in ammonium formate (1.5 M, pH 4.0) and subjected to analysis by InstantImager as described in Materials and Methods. Lane 1, VA13 cells treated with 100 μM cisplatin and lane 2, VA13 cells treated with 0.9 % saline only. (B) HPLC analysis was undertaken after 32P-postlabelling and one-dimensional TLC, as described in Materials and Methods. The top panel shows co-chromatography of 32P-postlabelled GpG and ApG synthetic standards, while the middle panel shows a representative chromatogram of DNA isolated from cisplatin-treated human cells. The lower panel shows control analysis of the labelling mix and deionized water.
Cisplatin. Cells were incubated for various times up to 24 h after this treatment prior to isolation of DNA and detection of intrastrand cross links by $^{32}$P-postlabelling. In the absence of p53, levels of both ApG and GpG cross links increased substantially within the first 2 h after cisplatin treatment, reaching maximal levels after 8 h (Figure 3). After 24 h, the levels of each adduct had decreased only slightly. GpG cross links were ~2.5 times the level of ApG cross links, in line with previous estimations of the proportion of each cross link formed in vitro (2). In an isogenic but p53-proficient cellular background (i.e. in the absence of tetracycline), maximal levels of ApG and GpG cross links were observed at lower levels than in the corresponding p53-deficient cells and at an earlier time point (between 0 and 2 h after treatment). After reaching this maximum, removal of both cross links proceeded rapidly with only low levels remaining after 8 h.

Identical experiments were performed in a second pair of cell lines: WI38 cells and their derivative cell line VA13 in which p53 is abrogated. ApG- and GpG-intrastrand cross links in p53-deficient VA13 cells reached maximum levels 2 h after cisplatin treatment, decreasing rapidly after 4 h. However, after this point, the remaining cross links persisted up to 24 h after treatment. In contrast, p53-proficient WI38 cells exhibited much lower maximum levels of both GpG and ApG cross links than the p53-deficient VA13 cells, decreasing by approximately half after 24 h.

**Discussion**

Previous work on the role of p53 in excision repair has principally focused on DNA damage induced by environmental agents such as UV, cigarette smoke and industrial waste (11–17). The implication from these studies has been that the loss of p53 increases genetic instability and reduces the ability of cells to process carcinogenic DNA lesions, thus increasing the risk of cancer. This study has focused instead on the use of a chemotherapeutic agent whose mode of action is dependent upon the persistence of the cytotoxic intrastrand cross links it induces.

We have established in this study that p53 is required for the global NER of the two major cisplatin-induced intrastrand cross links. Consistent with the studies of the repair of DNA damage induced by chemical carcinogens (15,17), removal of cisplatin-induced intrastrand cross links was offset by continued formation as a result of cisplatin remaining in the cells after treatment. Thus, p53-dependent NER is evident from reduced maximum levels of DNA damage in the first 2–4 h after treatment as well as a greater reduction in levels of DNA damage at later time points. p53-dependent removal of the cisplatin-induced intrastrand cross links was observed in two pairs of cell lines that differ in p53 status. In keeping with similar studies, in which the same cells were used to investigate the role of p53 in DNA damage-dependent cytotoxicity (26), the effect of p53 status on removal of the cross links was greatest in the 041TR cells and least evident for the ApG cross link in WI38 and VA13 cells. This is likely to result from the different mechanisms involved in altering p53 status in these cells and is consistent with other studies in which the use of viruses to abrogate p53 function in human cells elicited a limited impairment of p53-dependent NER in comparison to other model systems (13,14). Since the $^{32}$P-postlabelling approach allowed detection of individual DNA lesions formed by cisplatin rather than an assessment of total DNA damage, these results also extend host cell reactivation and atomic absorption-based studies which have revealed that the removal of platinum-induced DNA damage was more efficient in p53-proficient cells than p53-deficient cells (18,27) by monitoring the removal of the two major cisplatin-induced intrastrand cross links independently.
The $^{32}$P-postlabelling assay used in this study was based on the existing methods (21,22) for detection of cisplatin-induced DNA damage in in vitro-modified DNA, and adapted for the detection of cross links formed in human cell cultures. Further development of the assay will be needed in order to allow the accurate quantitation in cisplatin-treated cell cultures due to the large number of stages and low adduct recovery often observed in $^{32}$P-postlabelling procedures. However, in its current form the assay remains very useful for the determination of DNA repair profiles, even in the absence of absolute numbers of cross links, and was sufficiently sensitive to detect persistence and removal of cisplatin-induced cross links in different cellular backgrounds. Furthermore, it complements existing studies that have exploited the sensitivity and versatility of $^{32}$P-cellular backgrounds. It is also likely to be dependent upon the context of other p53-dependent pathways such as apoptosis. Since specific p53 mutations affect some pathways more than others (33), the biological and therapeutic response to cisplatin-induced DNA damage is also likely to be dependent upon the nature of the resulting functional p53 deficiency. Our own studies are continuing to evaluate the effect of specific p53 mutations on the cellular response to DNA damage, and which of these mutations are likely to have the greatest influence on the efficacy of DNA-damage-based chemotherapy.

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References


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