Copper uptake is required for pyrrolidine dithiocarbamate-mediated oxidation and protein level increase of p53 in cells

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The p53 tumour-suppressor protein is a transcription factor that activates the expression of genes involved in cell cycle arrest, apoptosis and DNA repair. The p53 protein is vulnerable to oxidation at cysteine thiol groups. The metal-chelating dithiocarbamates, pyrrolidine dithiocarbamate (PDTC), diethyldithiocarbamate, ethylene(bis)dithiocarbamate and H₂O₂ were tested for their oxidative effects on p53 in cultured human breast cancer cells. Only PDTC oxidized p53, although all oxidants tested increased the p53 level. Inductively coupled plasma MS analysis indicated that the addition of 60 μ M PDTC increased the cellular copper concentration by 4-fold, which was the highest level of copper accumulated amongst all the oxidants tested. Batho-

cuproinedisulphonic acid, a membrane-impermeable Cu(I) chelator inhibited the PDTC-mediated copper accumulation. Bathocuproinedisulphonic acid as well as the hydroxyl radical scavenger D-mannitol inhibited the PDTC-dependent increase in p53 protein and oxidation. Our results show that a low level of copper accumulation in the range of 25–40 μ g/g of cellular protein increases the steady-state levels of p53. At copper accumulation levels higher than 60 μ g/g of cellular protein, p53 is oxidized. These results suggest that p53 is vulnerable to free radical-mediated oxidation at cysteine residues.

Key words: Fenton, hydroxyl radical, thiol, tumour suppressor.

INTRODUCTION

The p53 tumour suppressor is a transcription factor that activates the expression of genes involved in growth arrest, DNA repair and apoptosis of the cell on genotoxic insult [1]. Stress-responsive cascades of enzymic activities regulate the DNA-binding function, stability and sub-cellular localization of p53. Its activity is regulated by post-translational modifications, including phosphorylation and acetylation.

Several protein kinases can phosphorylate p53 at serine and threonine residues after DNA damage [2–7]. Phosphorylation at the N-terminus of p53 regulates the transactivation function, by promoting interaction with the basal transcriptional machinery [8] and stability, via disrupting interaction with its negative-feedback regulator, murine double minute clone 2 oncoprotein (MDM2) [9]. Phosphorylation changes near the C-terminus of p53 induce a conformational change that stimulates DNA-binding [10,11]. The p53 protein becomes acylated near the C-terminus after DNA is damaged by two different histone acetyltransferases, namely p300 and P/CAF (p300/cAMP-response-element-binding protein) [12,13]. Phosphorylation of p53, in addition to acetylation, induces a conformational change that enhances its transactivation activity.

p53 is also vulnerable to oxidation at cysteine residues [14,15]. Analysis of its structure indicates that five cysteine residues (human Cys¹²⁴, Cys¹⁷⁶, Cys¹⁸², Cys²⁴², Cys²⁷⁷) are candidates for oxidation because of their location on the protein surface [15,16]. Although phosphorylation and acetylation of p53 primarily stimulate its activities, oxidation within its DNA-binding

domain seems to inhibit its activity. *In vitro*, recombinant p53 is very vulnerable to oxidation, which renders it incapable of binding DNA tightly [17,18]. In addition, p53 can bind copper to form Cu(I)-cysteinyl thiolates, rendering it incapable of binding DNA [19]. In cultured cells, treated with a metal-chelating compound, namely pyrrolidine dithiocarbamate (PDTC), p53 becomes oxidized at cysteine residues and cells become resistant to stress-induced up-regulation of p53 effector genes [20–22]. At present, it is unclear if other oxidants can oxidize p53 protein in cultured cells.

In the present study, we investigated the effects of three dithiocarbamate compounds PDTC, diethyldithiocarbamate (DEDTC) and ethylene (bis)dithiocarbamate (NABAM), as well as H₂O₂, on the oxidation state and level of p53 in cultured MCF7 cells. Here we demonstrate that, of the oxidants tested, only PDTC oxidizes p53. PDTC, DEDTC and H₂O₂ all increased the p53 level significantly, whereas NABAM did to a lesser extent. We provide results that strongly suggest that the oxidative and stabilization effect of PDTC on p53 is mediated by copper redox cycling via a Fenton-like reaction that generates free radicals. Our results demonstrate that PDTC oxidizes p53 and increases the p53 level in a copper-dependent and free radical-dependent manner.

EXPERIMENTAL

Cell culture

Human breast cancer MCF7 cells or A-1-5 rat embryo fibroblasts (gifts from Arnold J. Levine's Laboratory, Princeton, NJ, U.S.A.)

Abbreviations used: ALLN, *N*-acetyl-leu-leu-norleucinal; BCS, bathocuproinedisulphonic acid; DEDTC, diethyldithiocarbamate; DTT, dithiothreitol; MAL-PEG, methoxymaleimide polyethylene glycol; MDA, malondialdehyde; NABAM, ethylene (bis)dithiocarbamate; NEM, *N*-ethylmaleimide; PDTC, pyrrolidine dithiocarbamate; TBA, 2-thiobarbituric acid.

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were cultured in 90% Dulbecco's modified Eagle's medium (CAT No. 9031; Irvine Scientific) supplemented with 10% heat-inactivated foetal bovine serum (CAT No. 3000; Irvine Scientific) and penicillin-streptomycin (100 units/ml) (CAT No. BW04-649F; Fisher Biochemicals, Itasca, IL, U.S.A.) with 5% CO₂ at 37 °C.

Antibodies and reagents

The primary mouse monoclonal anti-p53 antibody DO-1 (CAT No. OP43-100UG) was purchased from CalBiochem (San Diego, CA, U.S.A.) and the secondary antibody, peroxidase-conjugated goat anti-mouse (CAT No. 115-035-008), from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). ECL[®] reagent (CAT No. RPN 2134) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). PDTC (CAT No. P-8165) was purchased from Sigma; diethylenetriaminepenta-acetic acid (CAT No. D-6518) from Sigma; DEDTC (CAT No. 7617) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.); NABAM (CAT No. 45593) from Sigma; H₂O₂ (30%; CAT No. 5240) from Mallinckrodt; bathocuproinedisulphonic acid (BCS; CAT No. 14,662-5) from Aldrich (Milwaukee, WI, U.S.A.); D-mannitol (CAT No. 423922500) from Acros Organic; *N*-acetyl-leu-leu-norleucinal (ALLN; CAT No. A-6185) from Sigma; *N*-ethylmaleimide (NEM; CAT No. E-1271) from Sigma; 2-thiobarbituric acid (TBA; CAT No. T-5500) from Sigma; dithiothreitol (DTT; CAT No. BP172-5) from Fisher; and methoxymaleimide polyethylene glycol (MAL-PEG, molecular mass 2 kDa; CAT No. 2D2M0D11) from Shearwater Polymer.

Detection of oxidized p53 in cultured cells

MCF7 cells were plated at 5×10^5 cells per 10 cm plate and grown in 10 ml of medium at 37 °C for 48 h before oxidant treatment. The medium was replaced with 5 ml of fresh medium containing the indicated concentrations of oxidants (PDTC: 0 or 60 μ M; DEDTC: 0, 60, 200 or 1000 μ M; NABAM: 0, 60, 200 or 1000 μ M; and H₂O₂: 0, 60, 100 or 500 μ M). ALLN (20 μ M), an inhibitor of the 26 S proteasome that degrades p53 after ubiquitination, was used as a control to amplify the p53 level. Methanol (1%), a solvent of the oxidants assayed, was tested for its background effect. For some PDTC treatments, the Cu(I) chelator BCS or the free-radical scavenger D-mannitol was added at the same time as PDTC. Cells were incubated at 37 °C for 5 h or for specified periods of time otherwise described and harvested by scraping with a rubber policeman into 2 ml of PBS (137 mM NaCl/2.7 mM KCl/4.3 mM Na₂HPO₄·7H₂O/1.4 mM KH₂PO₄). The cell suspension was centrifuged at 1500 *g* for 5 min, and the supernatant was removed. Cells were washed twice in 2 ml of PBS and stored at -80 °C for 1 h. Each cell pellet was thawed on ice and resuspended in 300 μ l of N₂-purged SEEN buffer [0.1 M sodium phosphate (pH 7.0)/5 mM EDTA/5 mM EGTA/0.1% Nonidet P40] supplemented with 1 mM PMSF, as a proteinase inhibitor and 80 mM NEM, as a capping agent of free thiol groups. The cell suspension was sonicated at 0 °C for 1 min and centrifuged at 2000 *g* for 10 min, and the supernatant was collected. The protein concentration was determined using a Bio-Rad assay (Reagent CAT No. 500-0006; Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA (CAT No. A-2056; Sigma) as the standard.

In vitro oxidation of p53

Recombinant human p53 was purified from baculovirus-infected Sf9 insect cells as described previously [23]. Purified p53 was eluted from a Q-Sepharose Fast Flow column in a buffer

containing 40 mM Tris/HCl, 0.4 M NaCl, 1 mM diethylenetriaminepenta-acetic acid and 10 mM DTT (pH 8.0). A total of 4 μ g recombinant human p53 (0.6 μ g/ μ l) was mixed with 2 mg of BSA (carrier protein) and dialysed in 1000 vols of Rexyn-treated 400 mM Tris/HCl (pH 7.3), 0.1% Nonidet P40 and 10 μ M DTT for 1 h 30 min at 8 °C. The protein mixture was divided into nine samples in duplicate and each sample was treated with different concentrations of CuSO₄ (0 or 20 μ M), H₂O₂ (0 or 500 μ M) and PDTC (0 or 60 μ M) in the presence or absence of BCS or D-mannitol. Sample mixtures were incubated on a rocking machine at 8 °C for 1 h 30 min, and 20 mM of NEM was added to stop the reactions. After 1 h incubation on ice, the samples were dialysed twice successively in 1000 vols of SEEN supplemented with 10 μ M of DTT for 1 h 30 min time intervals at 8 °C. Each sample was divided into two parts: one half was incubated with 20 mM DTT on ice and the other half was left untreated. Both DTT-treated and untreated samples were separately dialysed twice in 1000 vol. of SEEN for 1 h 30 min time intervals at 8 °C. All the samples were incubated with 500 μ M MAL-PEG on a rocking machine for 1 h at 8 °C and subjected to Western-blot analysis.

MAL-PEG tagging of oxidized cysteines

To remove excess NEM, each sample was dialysed twice successively for 3 and 12 h, in Slide-A-Lyzer[®] Mini Dialysis Unit (10 kDa molecular mass cut-off, CAT No. 69572; Pierce, Rockford, IL, U.S.A.) against 1000 vols of N₂-purged SEEN at 8 °C. The sample was equally divided into two aliquots, and one aliquot was incubated with 20 mM DTT at 0 °C for 1 h. The DTT-treated or untreated aliquot was separately dialysed twice successively, for 3 h and 9 h, against 1000 vol. of N₂-purged SEEN at 8 °C. To tag oxidized cysteine residues, all samples were incubated with 500 μ M MAL-PEG on a rocking machine at 8 °C for 1 h. After determining the cell protein concentration, 80 μ g of protein was resolved by SDS/10%-PAGE and transferred to Immobilon[™]-P membrane (CAT No. IPVH00010; Millipore, Bedford, MA, U.S.A.) for Western-blot analysis. The p53 primary antibody DO-1 was used at a dilution of 1:1000, and the secondary antibody, namely peroxidase-conjugated goat anti-mouse, was used at a dilution of 1:5000 as per the manufacturer's recommendations. The immunoblotted membrane was immersed in 6 ml of ECL[®] reagent for 5 min and exposed to Kodak Biomax ML film (CAT No. 862-5170). The MAL-PEG-tagged p53 was detected as a shifted protein on the blot. The membrane was then stained with Coomassie Blue dye to visualize actin as the indicator of overall protein level loaded in each lane.

Measurements of cellular metal concentrations

For measurements of the cellular copper and zinc concentrations after exposure to oxidants, cell extracts were prepared as described previously [21] with slight modifications. Cells in six 10 cm plates were treated under each chemical condition (mock, 100 μ M H₂O₂, 60 μ M PDTC, 60 μ M PDTC + 200 μ M BCS, 60 μ M DEDTC, or 60 μ M NABAM) for 5 h. After trypsinization, cells from three plates were mixed in a 15 ml centrifuge tube to obtain samples in duplicate. The cell suspension was centrifuged at 1500 *g* for 5 min, and the supernatant was removed. The cells were washed three times in 5 ml of 400 mM Tris (pH 7.3), treated with Rexyn resin (CAT No. R276-500; Fisher) to minimize exogenous metal contamination. The cell pellets were stored at -80 °C for 1 h. Each cell pellet was then thawed on ice and resuspended in 1 ml of 400 mM of Tris (pH 7.3, Rexyn-treated) supplemented with 0.1% Nonidet P40. The cell suspension was sonicated at 0 °C for 1 min and centrifuged at

2000 g for 10 min. The supernatant was collected as the whole cell lysate, and protein concentration was determined as the average of three measurements using Bio-Rad assay, with BSA as the standard to obtain 3–4 mg of protein/sample. The protein sample was diluted to 5 ml with 400 mM Tris (pH 7.3, Rexton-treated) supplemented with 0.1 % Nonidet P40 and stored at -20°C until the time of measurements. The amounts (ng) of copper and zinc in the total protein sample were measured using a Hewlett-PackardTM 4500 inductively coupled plasma MS with rhodium as the internal standard. Average values were determined from ten consecutive measurements (0.9 s integral time for each measurement). The concentrations of copper and zinc were calculated as μg of metal/g of protein.

TBA assay

The TBA assay was performed to detect the presence of lipid peroxidation products due to free radicals generated in cells, according to the method described previously in [24,25] with slight modifications. A-1-5 cells were plated at 5×10^5 cells per 10 cm-plate and grown in 10 ml of medium at 37°C for 48 h. Cells from two plates were treated with 5 ml of fresh medium containing 60 μM PDTC, and two other plates of cells were mock-treated. Cells were incubated at 37°C for 5 h and harvested by scraping in 2 ml of PBS. The cell suspension was centrifuged at 1500 g for 5 min, and the supernatant was removed. Cells were washed twice with 2 ml of PBS and stored at -80°C . Each cell pellet was thawed on ice, resuspended in 200 μl of PBS and refrozen at -80°C for 30 min. The cell suspension was thawed at room temperature, and 400 μl of 10 % (w/v) SDS in water was added to solubilize the membranes. Vortex mixing was performed for 5 min and the sample was incubated on a rocking machine for 30 min. The homogenate was transferred to a 10 ml glass test tube, and 1.5 ml of 20 % acetic acid, adjusted to pH 3.5 with 5 M NaOH, and 1.5 ml of 0.8 % (w/v) TBA were added. The tube was capped, and the cap was punctured for venting. The sample was incubated in a boiling-water bath for 1.5 h and cooled on ice for 1 min. TBA-reactive products formed a pink chromophore. Cleared supernatant of 1 ml was removed, and the absorbance was recorded at 532 nm. A mixture without cellular material was treated identically and was used to establish the baseline on the spectrophotometer.

RESULTS

Detection of oxidation and induction of the p53 protein after exposure to oxidant

Oxidative stress applied to the cell is one of the major factors that trigger the induction of p53 [26–33]. However, under certain conditions, p53 protein itself becomes oxidized and loses the wild-type conformation necessary for DNA binding [14,17,18,34]. Here, we examined the oxidative and inductive

Figure 1 Structures of oxidants used in the present study

PDTC, DEDTC, NABAM and H_2O_2 .

Figure 2 Detection of oxidized p53 by MAL-PEG tagging

First, free thiol groups are capped with NEM to prevent further thiol group reactions; secondly, oxidized cysteine residues are reduced by DTT; thirdly, the freed thiol groups are tagged with a large molecular-mass compound, MAL-PEG (2 kDa). MAL-PEG-tagged p53 corresponds to the oxidized form and is distinguished from the non-MAL-PEG-tagged (unoxidized) form as a shifted band on the Western-blot analysis.

effects of different dithiocarbamate compounds (PDTC, DEDTC and NABAM) and H_2O_2 on wild-type p53 in MCF7 cell cultures. The structure of each oxidant used in the present study is shown in Figure 1.

The method we used for detecting oxidized p53 in cultured cells is shown in Figure 2. On exposure to the oxidant, the oxidized form of p53 was detected by tagging oxidized cysteine residues with a large-molecular-mass compound MAL-PEG (with a dry molecular mass of 2 kDa). Tagged and untagged p53 were detected by Western-blot analysis (see the Experimental section for details). MAL-PEG tagging was specific to oxidized cysteine residues reducible by DTT, since all of the free thiol groups (i.e. reduced cysteines) were initially capped by NEM. MAL-PEG-tagged p53 corresponded to the oxidized form and was detected as higher-molecular-mass bands by monoclonal p53 antibody. Also, the p53 protein level was compared with samples from cells treated with and without oxidants.

Comparison of oxidative effects among different oxidants: PDTC oxidizes p53 and also increases the p53 protein level

The oxidative effects of different dithiocarbamate compounds as well as H_2O_2 on the p53 protein structure and level were compared. Treatment with 60 μM PDTC for 5 h significantly oxidized p53 in cultured MCF7 human breast cancer cells, as shown by the increased level of MAL-tagged p53 (Figure 3, lanes 7 and 8). The results also show that PDTC significantly increased the p53 level, suggesting that PDTC promotes a stress response within the cell. As both oxidized and unoxidized forms of p53 increased concomitantly in response to PDTC, it was necessary to confirm that the increase in the oxidized form was not simply due to the increase in the total p53 level that caused a higher molecular mass form of p53 to become detectable. A higher molecular mass form of p53 could be due to ubiquitin conjugation. To rule out this possibility, a synthetic peptide ALLN was used to increase the level of p53 by blocking its degradation by the 26 S proteasome [35]. ALLN treatment significantly increased the p53 level and led to the appearance of multi-ubiquitinated p53 proteins (Figure 3A). Importantly, ubiquitinated p53 did not migrate to the same position on the gel as the MAL-PEG-tagged p53. Furthermore, no oxidized p53 was detected after ALLN treatment, indicating that PDTC promoted p53 oxidation and did not simply increase the level of a pre-oxidized form of p53. Ubiquitinated p53 also became visible

Figure 3 The effect of PDTC, DEDTC and NABAM on p53 oxidation and level

MCF7 cells were exposed to DEDTC (**A**) or NABAM (**B**) for 5 h at indicated concentrations. The 26 S proteasome inhibitor ALLN and PDTC were used as positive controls for the protein level increase and oxidation of p53 respectively. Asterisks denote MAL-PEG-tagged forms of p53.

when the p53 level increased after oxidant exposure (Figure 3A) but it could further be distinguished from the MAL-PEG-tagged p53 by the fact that it was present in samples that had been processed without the DTT reduction step (MAL-PEG-conjugated forms of p53 are denoted with asterisks). Treatment with 60 μ M PDTC led to additional bands that corresponded to the MAL-PEG-tagged oxidized forms of p53 (Figure 3A). However, we did not consistently detect the highest molecular mass p53–MAL-PEG conjugate. These results confirmed that the appearance of the oxidized p53 on PDTC treatment was not due to an increase in a pre-oxidized form of p53, but, rather, due to actual protein oxidation by PDTC. Moreover, the results indicate that PDTC treatment consistently leads to oxidation of at least one site per p53 molecule.

We next explored if the closely related compound DEDTC could promote p53 level increase and oxidation. Treatment with

DEDTC at different concentrations (60, 200 or 1000 μ M) for 5 h did not cause significant oxidation of p53 in comparison with 60 μ M PDTC. Oxidized p53 became detectable only at high concentrations of DEDTC (> 200 μ M), but the level was much lower than that at 60 μ M PDTC (Figure 3A). Nevertheless, the same result shows that the increase in the p53 protein level by DEDTC even at the low concentration (60 μ M) was comparable with that detected after treatment with 60 μ M PDTC. The results indicate that DEDTC treatment at a high dose could oxidize p53, but only to a small degree compared with PDTC. At a low dose, DEDTC conferred some cellular stress that significantly increased the p53 protein level.

The treatment with NABAM in the range of 60–1000 μ M did not oxidize p53. Instead, NABAM led to a lesser increase of p53 protein level than that achieved with PDTC or DEDTC (Figure 3B). The results suggest that NABAM elicited no oxidation of

Figure 4 H_2O_2 increases the p53 protein level but does not oxidize it

MCF7 cells were exposed to H_2O_2 for 5 h at indicated concentrations (**A**) or for indicated periods of time at 500 μ M (**B**). The 26 S proteasome inhibitor ALLN and PDTC were used as positive controls for the protein level increase and oxidation of p53 respectively.

p53 protein and only a moderate stabilizing effect on p53. Next, a well-known oxidant H_2O_2 [26–28,33,36] was tested for its oxidative effects on p53 and compared with three dithiocarbamate compounds. Cells were treated with H_2O_2 either at different concentrations (60, 100 or 500 μ M) for the same period of time (5 h) or at the same concentration (500 μ M) for different periods of time (0.5, 1, 2 or 5 h) (Figure 4). H_2O_2 failed to oxidize p53 under any of these conditions, but it did cause the expected increase in p53 protein levels. This increase was dose-dependent, but treatment with 500 μ M H_2O_2 for 5 h increased the steady-state level of p53 protein to a lesser extent than that with 60 μ M of PDTC. Interestingly, 500 μ M H_2O_2 temporarily lowered the p53 protein level to below the basal level up to 1 h after treatment. Then, after 2 h, the protein level started to increase in a time-dependent manner (Figure 4B).

In summary, among the four oxidants tested, only PDTC significantly oxidized the p53 protein, although three compounds, PDTC, DEDTC and H_2O_2 increased the p53 protein level.

NABAM did not oxidize p53 and increased the p53 level to a lesser degree than the other compounds tested. Among the three dithiocarbamates, the oxidative and protein-stabilizing effects on p53 ranged from the highest, PDTC, to medium, DEDTC, and to the lowest, NABAM. Such differences may be attributed to different modes of cell permeation by these compounds.

Oxidative and inductive effects of PDTC on p53 are mediated by copper

We attempted to elucidate the mechanism of PDTC-mediated p53 oxidation and stabilization. Similar to other dithiocarbamates, PDTC chelates metal ions, particularly copper and zinc [37,38], suggesting that it may exert its effect on cellular functions via these redox-active metals. First, we investigated the increase in the copper and zinc concentrations in MCF7 cells after exposure to PDTC, in comparison with those cells exposed to DEDTC, NABAM and H_2O_2 . At 60 μ M, PDTC treatment

Figure 5 Measurement of cellular copper concentrations after treatment with oxidants

MCF7 cells were exposed to a different dithiocarbamates, namely PDTC, DEDTC or NABAM, at 60 μM or to H₂O₂ at 100 μM for 5 h, and the cellular concentrations of copper (A) and zinc (B) were measured using inductively coupled plasma MS. Membrane-impermeable Cu(I) chelator BCS, at a concentration of 200 μM, was used as an inhibitor of the PDTC-mediated copper import.

increased the cellular copper concentration by approx. 4-fold (Figure 5A). The membrane-impermeable Cu(I) chelator BCS was described previously to inhibit cellular responses to PDTC [37,39–41], so we tested for its inhibitory effect on the PDTC-mediated copper transport into the cell. Addition of 200 μM BCS to PDTC during incubation significantly suppressed copper import. DEDTC treatment also increased the copper concentration, but to a lesser degree (approx. 2-fold) than PDTC, whereas NABAM and H₂O₂ had essentially no impact on the cellular copper concentration. On the other hand, PDTC treatment only slightly increased the cellular zinc concentration (approx. 1.2-fold), and this small increase was not affected by the copper-specific BCS (Figure 5B). DEDTC, NABAM and H₂O₂ exposure did not significantly alter the cellular zinc concentration (Figure 5B). These results indicate that the cellular copper import by PDTC may be the major cause of its oxidative and stabilizing effect on p53.

To test this hypothesis, we asked if BCS could inhibit p53 oxidation and p53 protein level increase. As shown in Figure 6, addition of BCS (50 μM) to PDTC (60 μM) during cell incubation largely inhibited oxidation of p53. At BCS concentrations of 100 μM and above, p53 protein level increase was blocked. The result strongly indicates that copper is required for both p53 oxidation and p53 protein induction in response to PDTC. Interestingly, at concentrations of BCS greater than 100 μM in the presence of PDTC, the p53 level is very low. It is likely that in the absence of copper, PDTC has an inhibitory effect on p53 expression.

PDTC oxidizes and stabilizes p53 through a Fenton-like reaction

We next tested the hypothesis that PDTC, as the reactive Cu(I) complex, may undergo a Fenton-like reaction that generates an hydroxyl radical from intracellular H₂O₂. According to this scenario, the highly destructive hydroxyl radical can be the actual agent that oxidizes and stabilizes the p53 protein. If this is the case, free-radical scavengers should inhibit p53 oxidation and protein stabilization.

Figure 6 BCS inhibits the PDTC-mediated protein stabilization and oxidation of p53 in a dose-dependent manner

The effect of extracellular Cu(I) quenching by BCS on PDTC-mediated stabilization and oxidation of p53 was tested using MCF7 cells. Cells were incubated in the presence of PDTC, BCS or both PDTC and BCS for 5 h at indicated concentrations.

Figure 7 PDTC treatment increases free-radical production in cells

(A) The effect of D-mannitol, at concentrations ranging from 1 nM to 10 mM, on PDTC-mediated induction and oxidation of p53 was tested in MCF7 cells after 5 h of incubation. (B) A-1-5 cells were treated with PDTC and lipid peroxidation products were measured with the TBA assay as described in the Experimental section. Samples were prepared in duplicate. Absorbance readings were compared with a single sample that did not contain cells. Control (Ctrl) cells were vehicle-treated for 5 h prior to harvest. PDTC (60 μM), cells were treated with 60 μM PDTC for 5 h prior to treatment.

First, the free-radical scavenger D-mannitol was tested for its inhibitory effect on the PDTC-dependent oxidation and induction of p53 in MCF7 cells. Figure 7(A) shows that D-mannitol inhibits PDTC-mediated oxidation of p53. When D-mannitol concentration was 100 nM there was significant inhibition of p53 oxidation (lanes 13 and 14), and at 100 μM oxidation was completely blocked. D-Mannitol also inhibited PDTC-mediated stabilization of p53. Lower exposures of Western-blot analysis obtained from similar experiments indicate that in cells treated with PDTC plus 100 μM D-mannitol the p53 protein level is approximately the same as that in untreated cells (results not shown). In addition, we found that the use of the cell culture medium supplemented with pyruvate, another free-radical scavenger, attenuated the oxidative and protein stabilization effects of PDTC (results not shown). The results suggest that free radicals are necessary for PDTC-mediated p53 stabilization and p53 oxidation. The fact that low levels of D-mannitol prevent p53 oxidation but fail to prevent an increase in p53 level suggests that high levels of free radicals are necessary to oxidize p53. However, we cannot rule out the possibility that D-mannitol acts via another mechanism to prevent p53 oxidation. Although it is

unclear as to what this other mechanism could be, we note that two previous studies [42,43] have found that D-mannitol can induce apoptosis. Furthermore, the results demonstrate that direct p53 oxidation is not necessary for p53 level increase.

If PDTC generates free radicals, one would expect cellular lipids to become oxidized. One of the products of lipid peroxidation is malondialdehyde (MDA). We tested whether cellular MDA was generated in response to PDTC in a rat embryo fibroblast cell line known as A-1-5 cells. (We have previously shown that p53 in these cells was oxidized after treatment with PDTC [21].) PDTC treatment led to a 3.5-fold increase in MDA production compared with untreated cells (Figure 7B). These results indicate that the free radical is the actual effector of PDTC-mediated oxidation and induction of p53, and suggests that a Cu(I)-mediated Fenton-like reaction is the mechanism by which PDTC promotes its effect.

Copper can oxidize p53 *in vitro*

A Fenton reaction was created in the presence of p53 in an attempt to simulate the oxidative effects of PDTC-Cu observed

Figure 8 *In vitro* oxidation of recombinant p53

Purified recombinant human p53 was incubated at 8 °C for 8 h with or without CuSO_4 (20 μM), H_2O_2 (500 μM) and PDTC (60 μM) to promote oxidation of p53. BCS and mannitol were included, where indicated, to prevent p53 oxidation. p53 was then processed for detection of oxidation as described in the Experimental section.

in cell culture. Purified recombinant human p53 was incubated with CuSO_4 , and the effect on p53 oxidation was determined. As shown in Figure 8, p53 was oxidized after treatment with CuSO_4 , and this effect was abolished by BCS (cf. lanes 3 and 4 with lanes 5 and 6). This probably indicates that Cu^{2+} is capable of undergoing redox cycling under these conditions. Treatment of p53 with H_2O_2 promoted oxidation as well (Figure 8, lanes 7 and 8). Interestingly, we do not detect H_2O_2 -induced p53 oxidation in cells, indicating that the cell has adequate defences to prevent H_2O_2 -mediated oxidation. PDTC treatment led to a very low level of oxidation of p53 (Figure 8, lanes 9 and 10). Treatment with PDTC and CuSO_4 does not seem to alter the level of oxidation produced by CuSO_4 alone (cf. lanes 17 and 18 with lanes 3 and 4). This indicates that PDTC has no effect on p53 oxidation *in vitro*. On treatment with the three compounds CuSO_4 , PDTC and H_2O_2 , the p53 protein is also highly oxidized as indicated by the presence of two higher molecular mass bands (lanes 21 and 22). Free radicals appear to be involved in oxidation of p53 because D-mannitol is able to prevent oxidation of the p53 protein (lanes 25 and 26). The conclusion from this experiment is that Cu^+ is sufficient to oxidize p53 via a mechanism that requires free radicals. It should be noted that breakdown of p53 protein accompanied its oxidation as evidenced by lower molecular mass bands on the Western-blot analysis (results not shown). This is probably due to free radical-induced cleavage of the peptide bond. The diminution of the signal of the oxidized p53 forms may also be due to partial occlusion of the p53 antibody by the MAL-PEG conjugate in the Western analysis. The results demonstrate that a copper-dependent free-radical reaction can lead to p53 oxidation.

DISCUSSION

We have demonstrated that PDTC requires copper to oxidize p53 and to increase the p53 protein level in cultured breast cancer cells expressing wild-type p53. However, oxidation may not be required for p53 protein level increase. Other structurally similar dithiocarbamates DEDTC and NABAM also increase the level of p53 protein but fail to oxidize the protein. Among the

dithiocarbamates tested, PDTC is the most efficient in accumulating copper into cells. Under normal circumstances, copper chaperones such as metallothionein and ceruloplasmin chelate copper, which prevents the release of free copper atoms in biological systems [44]. However, owing to defects in the copper transport pathway, there are rare diseases such as Wilson disease and haemochromatosis where copper accumulates in the liver [45]. In such cases, intracellular proteins with accessible cysteine thiol groups may be susceptible to oxidation. Interestingly, a recent report has shown that copper ions increase the level of p53 and promote p53 nuclear accumulation in cultured liver cells [46]. These results are consistent with the effects on p53, which we detected using PDTC in cultured cells [22].

It is likely that copper loading has multiple effects on the p53 signalling pathway. According to our result, dithiocarbamate-mediated accumulation of copper over the range of 25–40 $\mu\text{g/g}$ of cellular protein increases the steady-state level of p53. Stabilization of p53 is apparently the result of free-radical formation, which can promote DNA backbone-strand breaks [47]. DNA-strand breaks trigger p53 phosphorylation by kinases known as ATM and checkpoint kinase 2 (CHK2) and lead to an increase in p53 protein level by inhibition of MDM2-mediated p53 degradation [48,49]. The low level of copper necessary for genotoxic stress-induced p53 activation is consistent with the fact that a p53 response can be elicited when less than ten DNA double-strand breaks occur per cell [50]. At levels of copper poisoning higher than 60 $\mu\text{g/g}$ of cellular protein, such as those mediated by PDTC in the present study, proteins with exposed thiol residues can become oxidized. At the concentrations used in this study, DEDTC, NABAM and H_2O_2 probably create a sufficient amount of free radicals to promote DNA-strand breaks and increase p53 protein concentration. Only PDTC is able to produce a sufficient amount of free radicals to oxidize p53 protein.

Although PDTC is a well known Cu(II) chelator, the present study shows that a membrane-impermeable Cu(I)-specific chelator prevents PDTC-mediated copper accumulation. This suggests that Cu(II) appears to be reduced to Cu(I) prior to its transport. Two classes of plasma membrane-associated enzymes

are known to reduce Cu(II) to Cu(I), namely the surface NADH oxidases [51] and the metalloredutases encoded by the *FreI–Fre7* genes [52,53]. Extracellular production of Cu(I) is also necessary for cell killing, as evidenced by the fact that BCS prevents PDTC-mediated neuronal cell death [40]. Thus it is probable that Cu(I) is produced extracellularly and it migrates into the cell. Cu(I) may be transported by the Cu(I) plasma membrane transporter CTR [54]. PDTC may efficiently present Cu(II) to the reductase enzymes on the surface of the protein. In addition, PDTC may itself act as an efficient Cu(I) ionophore due to its lipophilicity [55].

With the technique of MAL-PEG tagging, we consistently detected one major site of oxidation. Previously, we have shown that MAL-PEG tagging is extremely efficient in labelling cysteine thiol groups in the model protein rabbit skeletal-muscle creatine kinase [23]. But we cannot rule out the possibility that more cysteine residues on p53 are oxidized than those indicated by MAL-PEG tagging. MAL-PEG tagging of one oxidized cysteine residue may sterically hinder the tagging of a second oxidized cysteine residue. According to the solved p53 crystal structure, we note that three of the six cysteine sulphur atoms on the surface of the p53 protein molecule (Cys¹⁸², Cys¹⁷⁶, Cys²⁴²) lie within a linear distance of 13 Å (1 Å ≡ 0.1 nm) from each other [15,16]. It is possible that MAL-PEG tagging at one of these sites may preclude MAL-PEG tagging of the other sites. Furthermore, if the p53 thiol groups were oxidized to higher oxidation states (sulphinic acid or sulphonic acid) our MAL-PEG tagging technique would not be able to detect the oxidation due to the irreversibility of the oxidation reaction. Efforts to map the sites of p53 oxidation are in progress in our laboratory.

The mechanism of p53 cysteine thiol group oxidation is probably mediated by Fenton chemistry. In other words, H₂O₂ is reduced by a single electron to form a hydroxyl radical and a hydroxide ion. Our results show that PDTC treatment leads to a substantial increase in the lipid peroxidation product MDA, which is a major product of hydroxyl radicals. We did find that D-mannitol was able to block oxidation of p53. However, only a low concentration of this radical scavenger is needed to block oxidation, suggesting that it may bind to specific sites on the cell surface where Cu²⁺ undergoes reduction. Further studies are required to understand more clearly the mechanism by which D-mannitol prevents p53 oxidation. A previous study [19] showed that p53 can bind Cu(I); although the site of Cu(I) binding remains unclear, it may be that the p53 thiol groups that bind copper become oxidized. When cellular proteins are treated with oxidizing agents, their cysteine thiols form sulphenic acid or form mixed disulphides with glutathione [56,57]. Since we could promote p53 oxidation *in vitro* and in the absence of glutathione, it is certainly possible that p53 cysteine residues form sulphenic acids. However, this does not rule out the possibility that p53 may undergo a different type of oxidation reaction *in vivo*. Future studies will indicate if pathological conditions exist where redox-sensitive metals oxidize p53.

We thank Susan Kane (City of Hope National Laboratory at Duarte, CA, U.S.A.) for suggestions on the paper. This work was supported by the Minorities in Biomedical Research Sciences Program (NIGMS08101) and the Bridges-to-the-Future Program (NIGMS49001).

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Received 30 August 2001/5 April 2002; accepted 19 April 2002

Published as BJ Immediate Publication 19 April 2002, DOI 10.1042/BJ20011251