Cooperation between p53 and hMLH1 in a Human Colocarcinoma Cell Line in Response to DNA Damage[1]

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ABSTRACT

We have studied the possible interactions between the mismatch repair system and p53 in a human colon cancer cell line, HCT-116 (known to have a homozygous mutation in mismatch repair gene *hMLH1* on chromosome 3) and in a clone obtained after insertion of a single copy of chromosome 3 (HCT-116+ ch3).

Loss of DNA mismatch repair activity resulted in resistance to cisplatin (DDP). p53 accumulated differently in these cell lines after treatment with DDP. Initially at similar high levels after DDP treatment, p53 maintained the increase in HCT-116 cells, even 72–96 h after drug exposure, whereas HCT-116+ch3 mismatch-proficient cell line p53 declined to basal levels after 48 h. The higher levels of p53 in mismatch-deficient HCT-116 cells were accompanied by increased transcriptional activity as assessed by the gel-retardation assay and by activation of a promoter containing a p53 DNA binding site.

To better understand the role of p53, if any, in cell sensitivity to DDP, we disrupted p53 in both cell lines by stable transfection with the human papillomavirus type 16 *E6* gene. HCT-116/E6 cells were more sensitive to DDP than the parental cell line, whereas HCT-116+ch3/E6 were fairly similar to HCT-116+ch3 with normal p53 function.

Although in our system the transfer of the entire chromosome 3 was used (thus not excluding a possible role of other genes localized on this chromosome), our data indicate that p53 can cooperate with the mismatch repair system. In fact, the lack of *hLMH1*, at least in these cells, enhances the role of p53 in protecting the cells from DDP-induced DNA damage.

INTRODUCTION

DDP[3] is an anticancer agent presently used for the treatment of a variety of tumors. The efficacy of treatment is often limited by the onset of resistance mechanisms (1, 2). Resistance to DDP is multifactorial and recently has also been related to mismatch repair deficiency both *in vitro* and *in vivo* (3–6). A correlation between defects in the mismatch repair system and resistance to DDP has been shown in colon cancer cells HCT-116 (6) and in the ovarian cancer cell line A2780 (7). DDP resistance conferred by loss of mismatch repair activity may be due to failure of this DNA damage detector system to recognize the lesion and to activate a pathway leading to apoptosis (8).

There are also many new findings on the relation between p53 status and cell sensitivity to DNA-damaging agents, particularly DDP. The data thus far are contradictory and show a protective role of p53, independence from the p53 status, or increased sensitivity in the presence of wild-type p53 (9–15) to different chemotherapeutic agents. Possible connections between sensitivity to DDP, p53 status, and mismatch repair capacity are suggested by the finding that in cancer cells, long-term selection with DDP created not only resistance to this agent but also loss of hMLH1 functions and loss of p53 activity (5).

The aim of the present work was to see whether the absence of mismatch repair activity influenced the functions of p53 in a well-defined cellular system after the induction of DNA damage with DDP.

MATERIALS AND METHODS

Cell Culture, Stable Transfectants, and Drug. The hMLH1-deficient human colorectal adenocarcinoma cell line HCT-116 and the subline into which a wild-type copy *hMLH1* on chromosome 3 has been introduced by microcell fusion (HCT-116+ch3) were obtained from Dr G. Marra (Zurich, Switzerland). Both cell lines [the growth and molecular characteristics of which have been described (16)] were maintained in Iscove's modified Dulbecco medium (Sigma Chemical Co.), supplemented with 100 mM L-glutamine and 10% fetal bovine serum. The chromosome-complemented cell line was grown in medium with 400 μg/ml of G418 (Sigma). Experiments were performed in medium without G418.

Expression of hMLH1 was documented by Western blot for each cell line (data not shown). For stable transfection, HCT-116 and HCT-116+ch3 were seeded 24 h before transfection with pCMV-16E6 plasmid (kindly provided by Dr. Kathleen Cho, The Johns Hopkins, Baltimore, MD) using calcium

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[3] The abbreviation used is: DDP, cisplatin.

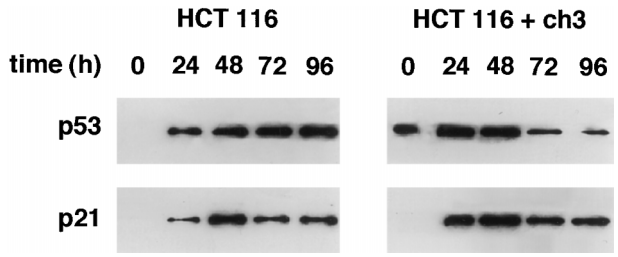

Fig. 1 p53 and p21 activation in response to DDP. Cells were exposed to DDP (25 μM for HCT-116 and 12.5 μm for HCT-116+ch3) for 1 h. The cells were tested at the times indicated from the beginning of exposure. p53 and p21 proteins were immunodetected with antibodies to p53 and p21.

phosphate precipitate. HCT-116+ch3 cells were cotransfected with plasmid pLSXH containing the hygromycin resistance gene. Clones were selected on 400 μg/ml of G418 and 80 μg/ml of Hygromycin B (Sigma). After expansion, colonies were selected and tested for p53 function after X-irradiation, using a Philips 6 MV X-ray source at 3 Gy/min.

DDP was obtained from Bristol Meyers-Squibb (Wallingford, CT) and was dissolved in culture medium just before use. For clonogenic assay, cells were plated at 1000 cells/cm^2, treated with different concentrations of DDP for 1 h, and incubated for 10–14 days in drug-free medium, and the colonies formed (~50 cells) were counted after staining with Giemsa.

Flow Cytometry. Cells (2×10^6) were removed 24 h after treatment with DDP or X-rays, washed twice in ice-cold PBS, fixed in ice-cold 70% ethanol, washed in PBS, treated with RNase (500 units/ml; Sigma) at 37°C for 30 min, and stained with propidium iodide (50 μg/ml; Sigma). Cell cycle analysis was done with a Coulter fluorescence-activated cell analyzer (Becton Dickinson, Mountain View, CA) with a minimum of 10,000 events, by the Multicycle program provided by the manufacturer, and data were expressed as the percentages of cells in G_1, G_2, and S phases.

Protein Extraction and Western Blotting Analysis. Cells were collected at different time points after treatment with DDP, and proteins were extracted according to procedures described previously (17). Samples were lysed on ice for 30 min in 0.1% NP40, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 50 mM NaF in the presence of aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. For each sample, 30 μg of proteins were electrophoresed through 12% SDS-PAGE and transferred to nitrocellulose filters. The filters were hybridized with antibodies against p53 (DO-1) and p21 (both from Santa Cruz Biotechnology, Santa Cruz, CA) and detected with the ECL system (Amersham, UK).

Transcriptional Activity. For this assay, we transiently transfected 5 μg of pG13Luc, which contains 13 copies of the p53 DNA binding site cloned upstream of the luciferase gene. To eliminate potential differences due to transfection efficiency, 2 μg of β-galactosidase expression vector RSV-lac was included in all transfections. Luciferase was detected in cell extracts using the Luciferase assay system (Promega, Madison, WI).

Gel Retardation. For the gel retardation assay, a double-stranded oligonucleotide containing the p53 consensus DNA binding site was 5′ end labeled with T4 polynucleotide kinase and [^{32}P]γATP. In a final volume of 25 μl, 1 ng (30,000–40,000 cpm) of oligonucleotide was incubated for 30 min at 0°C with 27 μg of total protein extracts in the presence of 3 μg of poly(deoxyinosinic-deoxycytidylic acid), 150 ng of anti-p53 monoclonal antibody (pAb 421; Santa Cruz Biotechnology). Free and protein-bound DNA were separated on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. Specificity of p53 binding was checked by competition with a 50-fold molar excess of the same, unlabeled oligonucleotide.

RESULTS

Activation of p53 and p21 in response to DDP Treatment. HCT-116 and HCT-116+ch3 cells were exposed for 1 h to concentrations of DDP causing similar inhibition of cell growth in both lines, then the cells were incubated in drug-free medium for various times; cell lysates were analyzed by Western blotting (Fig. 1). Steady-state p53 levels were, in some experiments, higher in HCT-116+ch3 than in parental HCT-116 cells; however, DDP was able to induce p53 and p21 in both lines. The effect was visible and similar in the two lines 24 h after treatment. However, the increase in the amount of p53 persisted in HCT-116 cells up to 96 h after drug washout, whereas by the same time p53 had returned to basal levels in HCT-116+ch3 cells.

We checked whether the increased stability of p53 in HCT-116 cells corresponded to an increased transcriptional activity using two approaches: the transfection of plasmids encoding for luciferase under the control of p53 (Fig. 2*A*), and the gel retardation assay (Fig. 2*B*). Both systems independently showed that the lasting increase in the amount of p53 in the HCT-116 cell line reflected greater p53 transcriptional activity and correlated with the induction of p21 observed in these cells.

Characterization of HCT-116 and HCT-116+ch3 Cells with Disrupted p53 Functions. The degree of inactivation of p53 functions was evaluated in different clones selected from HCT-116 and HCT-116+chr3 cell lines after transfection with HPV-E6 after classical induction of DNA damage by X-irradiation (Fig. 3). Two clones were selected (N7 and N8), which had a pronounced defect in p53 function, as indicated by the absence of p53 accumulation and of induction of G_1 arrest after X-irradiation (Fig. 3). The same holds true for other clones (data not shown). Additional studies were conducted only in clone N7 (from HCT-116) and clone N8 (from HCT-116+chr3).

Disruption of p53 Functions, Cell Sensitivity, and Cell Cycle Progression by DDP. The HCT-116/E6 clone with disrupted p53 function was more sensitive to DDP than parental cells (Fig. 4) (IC_{50} ~5-fold lower, 5 μM *versus* 25 μM). In contrast, disruption of p53 in HCT-116+ch3 cells did not significantly alter cell sensitivity (IC_{50}, 15 and 18 μM, respectively, for HCT-116+ch3 and HCT-116+ch3/E6 cells).

We tested the ability of DDP to activate p53 in these cells (Fig. 5). As already observed after irradiation, after DDP treatment clear p53 induction was seen in the two parental cell lines, whereas the p53 levels in the E6-transfected clones did not change after treatment. These increased p53 levels were able to bind DNA, as evidenced by gel retardation assay (*lower panel*)

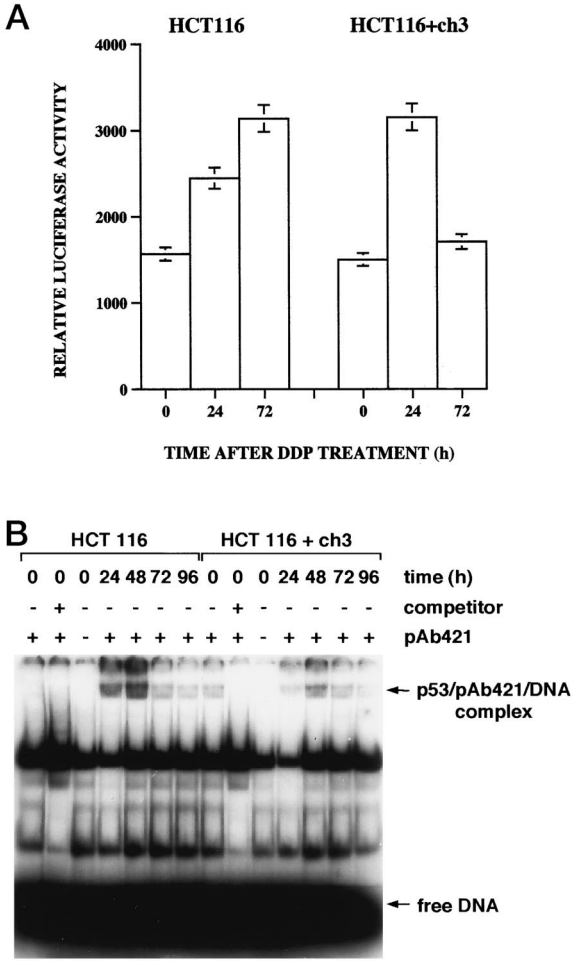

Fig. 2 Transcriptional activity of p53 in HCT-116 and HCT-116+ch3. Cells were untreated or treated with DDP (25 μM for HCT-116 and 12.5 μM for HCT-116+ch3) for 1 h and transfected with 5 μg of pG13Luc and 2 μg of RSV-gal (*A*). Luciferase activity was measured after 24 h and normalized to β-galactosidase activity. Transfections were done at the end of DDP treatment or after 72 h of incubation in drug-free medium. Data are means of at least three experiments performed in duplicate; *bars*, SD. *B*, nuclear proteins were analyzed for p53 binding to ^{32}P-labeled oligonucleotide in the presence of pAb421 antibody. Proteins were isolated at 24 or 72 h after DDP treatment.

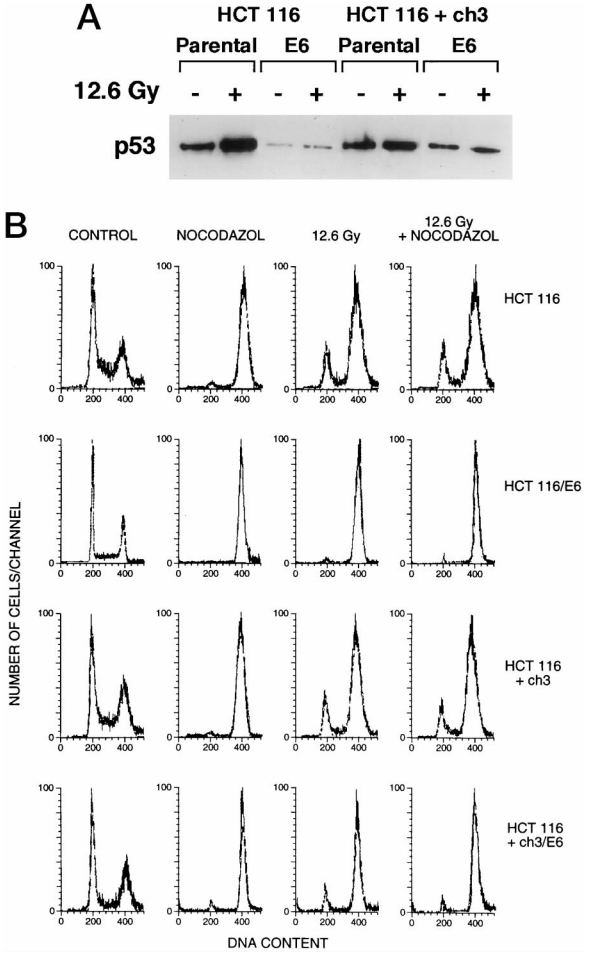

Fig.3 Characterization of HCT-116 and HCT-116+ch3 cells with intact and disrupted p53 functions. *A*, exponentially growing cells (−) were subjected to 12.6 Gy of X-rays, and 1.5 h after irradiation (+), cells were prepared for Western blotting. Immunodetection of p53 protein was done with antibody to p53. *B*, exponentially growing HCT-116 and HCT-116+ch3 were irradiated with 12.6 Gy of X-rays and then incubated for 16 h in the presence or absence of the mitotic inhibitor nocodazole (0.4 μg/ml). Cell cycle progression was assayed by flow cytometry.

in which an increased p53/DNA complex was observable after drug treatment in HCT116 and HCT-116+ch3 cells but not in E6-transfected clones.

DDP-induced accumulation of cells in G_2 phase of the cell cycle was determined by flow cytometry. Incubation of the parental cell line HCT-116 with DDP resulted in a limited accumulation of cells in G_2, with a ratio between the percentage of cells in G_2 and G_1 (G_2:G_1) of 3.4. Incubation of HCT-116/E6 cells with the same dose of DDP resulted in marked accumulation of cells in G_2 with a G_2:G_1 ratio of 8.6. At the same time, in HCT-116+ch3/E6 cells, DDP caused a small change in G_2 accumulation (G_2:G_1 ratio 1.3) in comparison with DDP-treated parental HCT-116+ch3 cells (G_2:G_1 ratio, 1.9). The minor differences in cell cycle distribution observable in the latter system correlate well with the data obtained on cell sensitivity.

DISCUSSION

Mismatch repair deficiency has been shown previously to confer resistance to the cytotoxic activity of simple methylating agents such as methylnitrosourea and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (18, 19). The loss of DNA mismatch repair also results in constitutive resistance to DDP in human cancer cells, although to a lower extent (6,12). The current hypothesis is that DNA mismatch repair is needed as a detector of DNA damage and that drug resistance, particularly to DDP, results from the cells failing to recognize DDP-DNA adducts and activate signaling pathways to trigger apoptosis. Details of the mechanisms, however, are far from clear.

It is established, however, that DDP-induction of DNA damage leads to many events, one of which is increased activity of the p53 protein (20). p53 either mediates growth arrest, both in G_1 or G_2 phases of the cell cycle, or directs cells to apoptosis.

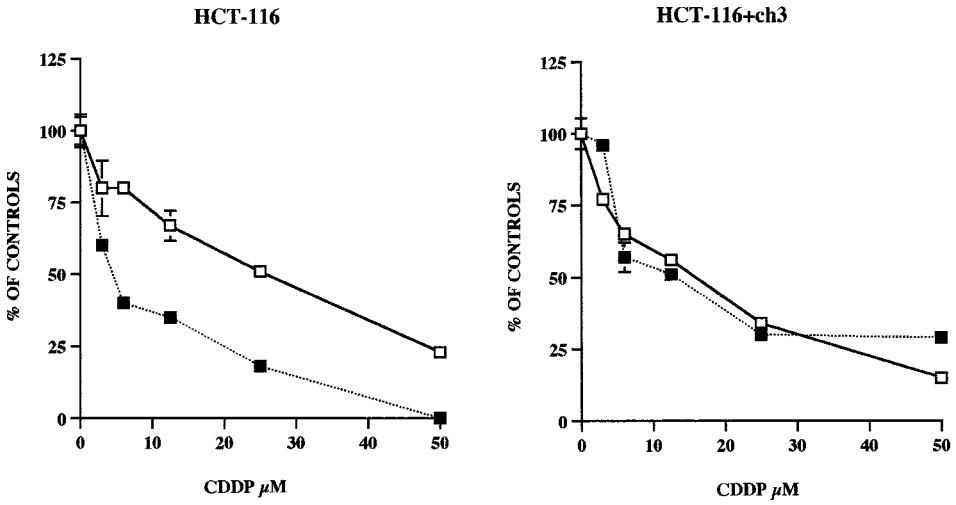

Fig. 4 Effect of DDP (*CDDP*) on survival of HCT-116 and HCT-116+ch3 with intact and disrupted p53. Values are means of three experiments, each consisting of five replicates; *bars*, SD.

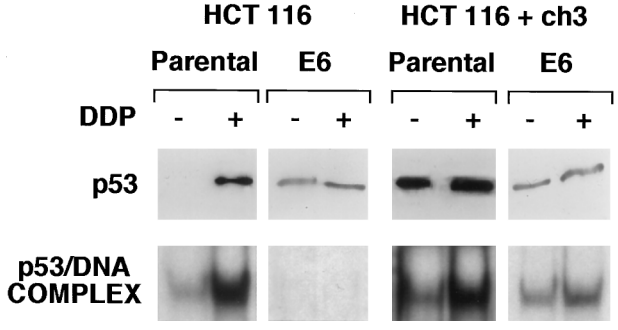

Fig. 5 Western blotting analysis of p53 expression (*upper panel*) and formation of p53/DNA complex by gel retardation assay (*lower panel*). Cells were exposed for 1 h to DDP (25 μM for HCT-116 and 12.5 μM for HCT-116+ch3) and tested after 24 h.

It has been suggested that these two cellular decisions are distinctive end points of p53 induction, depending on the cellular context and the type of DNA damage (21–24).

We investigated whether p53, which plays such an important role in cell sensitivity to DDP, was a partner of the mismatch repair system at least in this cellular context, where this system seems to be responsible for the constitutive DDP resistance. Our results show that the absence of DNA mismatch repair due to loss of *hMLH1* leads to an increase in p53 stability after DNA damage. These data corroborate previous observations in clones derived from the human ovarian carcinoma cell line A2780, which are defective in mismatch repair (25). Recent data, obtained in a similar system, indicate possible independent pathways of p53 induction by X-rays and DDP (26). The persisting high levels of p53 in HCT-116 after induction of DNA damage correlated with an increase in its transcriptional activity, indicating that the protein is functional.

E6-mediated disruption of p53 function in HCT-116 cells sensitizes these cells to DDP. This finding confirms previous observations made in an independently obtained HCT-116/E6 clone (10). The increased DDP sensitivity of HCT-116/E6 cells, compared with the parental HCT-116 cell line, is associated with an increased accumulation of cells in the G_2 phase of the cell cycle, a finding also reported for other compounds in different cell systems (10, 27).

We did not observe any real differences in DDP-induced cytotoxicity after p53 disruption in HCT-116+ch3 (mismatch-repair proficient cells), which suggests that in the presence of an efficient mismatch repair system, p53 does not influence the cell sensitivity as strongly as in mismatch repair-defective lines. Although in this experimental system the entire chromosome 3 has been transferred in HCT-116 cells (thus not excluding the possibility that other genes, besides *hMLH1*, in this chromosome could interact with p53), the results here obtained are in agreement with the data reported in the literature in other cellular systems. It has in fact been reported that transfection of a dominant mutant p53 in A2780 cells (mismatch repair proficient) does not significantly change DDP-induced cytotoxicity, whereas the same dominant mutant transfected in a clone derived from A2780 (CP70, mismatch repair deficient) induced a significant increase in DDP activity (28). In addition, by comparing E6-transfected clones obtained from HCT-116 or MCF-7 cells (9, 10), the results are that disruption of p53 in mismatch repair-deficient cells (HCT-116) greatly enhances sensitivity to DPP whereas in MCF-7 [apparently mismatch repair proficient (5)], the effects were much lower (9).

In summary, our results indicate that p53 can cooperate with the mismatch repair system because when the latter is deficient, p53 protects at least some types of cells against death induced by DNA-damaging anticancer agents.

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