Translesion Polymerase η Is Upregulated by Cancer Therapeutics and Confers Anticancer Drug Resistance

Maja T. Tomicic, Dorthe Aasland, Steffen C. Naumann, Ruth Meise, Christina Barckhausen, Bernd Kaina, and Markus Christmann

Abstract

DNA repair processes are a key determinant of the sensitivity of cancer cells to DNA-damaging chemotherapeutics, which may induce certain repair genes as a mechanism to promote resistance. Here, we report the results of a screen for repair genes induced in cancer cells treated with DNA crosslinking agents, which identified the translesion polymerase η (PolH) as a p53-regulated target acting as one defense against interstrand crosslink (ICL)-inducing agents. PolH was induced by fotemustine, mafosfamide, and lomustine in breast cancer, glioma, and melanoma cells in vitro and in vivo, with similar inductions observed in normal cells such as lymphocytes and diploid fibroblasts. PolH contributions to the protection against ICL-inducing agents were evaluated by its siRNA-mediated attenuation in cells, which elevated sensitivity to these drugs in all tumor cell models. Conversely, PolH overexpression protected cancer cells against these drugs. PolH attenuation reduced repair of ICL lesions as measured by host cell reactivation assays and enhanced persistence of γH2AX foci. Moreover, we observed a strong accumulation of PolH in the nucleus of drug-treated cells along with direct binding to damaged DNA. Taken together, our findings implicated PolH in ICL repair as a mechanism of cancer drug resistance and normal tissue protection. Cancer Res; 74(19); 5855–96. ©2014 AACR.

Introduction

Because of the high toxicity in replicating cells, genotoxic agents are being used as anticancer drugs. Most important are drugs that induce DNA interstrand crosslinks (ICL), which are potent killing lesions (1). The ICL-inducing drugs include derivatives of nitrogen mustards and chloroethyl nitrosoureas. One of the most often used nitrogen mustard is cyclophosphamide, whose active metabolite alkylates the N7 position of guanine yielding cytotoxic ICL (2). Chloroethyl nitrosoureas used in cancer therapy are carmustine (BCNU), lomustine (CCNU), nimustine (ACNU), and fotemustine (Maphoran). Upon formation of a nucleophilic chloroethylheterenium ion, the O6-position of guanine becomes alkylated, leading to the formation of O6-chloroethylguanine (O6-CIG; ref. 3). O6-CIG can be repaired by the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT; ref. 4). If not repaired, O6CIG undergoes intramolecular rearrangement to form N1-O6-ethenoguanine and finally N1-guanine-N3-cytosine ICLs (5, 6). O6-chloroethylating agents are being used for the treatment of glioblastoma, astrocytoma, and, albeit to a lesser extent, malignant melanoma, gastrointestinal, and pancreatic cancer. Hodgkin and non-Hodgkin lymphoma (7). Fotemustine is approved for the treatment of metastasizing melanoma and shows improved response rates and an increased survival over dacarbazine in the treatment of disseminated cutaneous melanoma (8). Also, it showed promising results in combination with ipilimumab (9), bevacizumab (10), and temozolomide (11).

Tumor cells are able of counteracting the killing effects of these anticancer drugs, using various strategies including DNA repair (12, 13). Activation of DNA repair by anticancer drugs occurs on various levels, which includes the transcriptional activation of repair genes (14). An important factor involved in transcriptional regulation of DNA repair genes is p53, which becomes activated via the ATM/ATR pathway by DNA-damaging agents and ionizing radiation that cause DNA replication arrest and DNA double-strand breaks (DSB; for review, see ref. 15). As a consequence, the cellular amount of p53, its nuclear translocation, and its DNA-binding activity are enhanced and target genes become transcriptionally activated (16). Activation of p53 results in cell-cycle arrest, which occurs either in the G1 or G2–M phase (17) and upregulation of repair genes (14), thereby causing resistance to anticancer drugs.

In glioblastoma cells, p53 mediates resistance against the topoisomerase I inhibitors, topotecan and irinotecan (18–21) and the chloroethylyating agent ACNU (22). In case of ACNU, the protective effect was shown to be the result of p53-driven induction of the nucleotide excision repair genes xpc and ddh2 (22). Protection by p53 against chloroethylating agents was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
also observed in melanoma cells, where it was shown to trigger resistance against the anticancer drug fotemustine (23). In contrast, p53 sensitizes melanoma cells to cisplatin by activation of apoptosis (24).

To identify p53 target genes involved in DNA repair that contribute to protection of melanoma cells against chloroethylating agents, qRT-PCR arrays were performed, analyzing the expression of all known DNA repair genes upon fotemustine treatment of D05 melanoma cells wild-type for p53. Besides the well-characterized p53 targets ddb2 and xpc (25), a strong and robust induction of the translesion polymerase η (PolH) was observed. PolH plays an important role in tolerating replication blocking DNA lesions by translesion synthesis (TLS; ref. 26) and is also involved in the replication at fragile sites in the absence of exogenous stress (27, 28). Here, we show induction of PolH following treatment with ICL-inducing drugs, which occurs in p53 wt but not p53-mutated tumor cells. It was also observed in nontransformed cells and in a mouse xenograft model in vivo. Furthermore, we show the induction contributes to resistance against all tested ICL drugs: fotemustine, lomustine (CCNU), and the cyclophosphamide analogon mafosfamide. The protective effect results from involvement of PolH in the repair of DNA damage induced by these drugs. The data provide an example of acquired drug resistance by upregulation of a TLS polymerase. As upregulation of PolH was found in normal and cancer cells, the data have implications both for cancer cell resistance and protection of the normal tissue.

Materials and Methods

Cell lines and anticancer drug treatment

The cell lines used (A375, D03, D05, MCF7, TK6, U87, LN229, U138, T98G, LN18, LN308, LN319, VH10(ert)) were described previously (23, 25, 29, 30) and grown in RPMI or DMEM medium containing 10% FBS, in 7% CO2 at 37°C. Fresh blood was obtained from healthy donors obtained from the blood bank of the University Medical Center Mainz (Mainz, Germany). Peripheral blood lymphocytes (PBLC) were isolated using density centrifugation and Lymphoprep (PROGEN Biotechnik GmbH) as described (29). For growth stimulation, lymphocytes were incubated for 24 hours in 24-well tissue culture plates containing plastic-bound anti-CD3 (BD Pharmingen) plus anti-CD28 antibodies (BD Pharmingen). If not differently stated, cells were pre-exposed to 10 μmol/L O6-benzylguanine (O6BG) and one hour later exposed to fotemustine (diethyl-3-(2-chloroethyl)-3-nitrosoureido)ethylphosphate; Muphoran, Servier Research International), CCNU (Sigma Aldrich), or mafosfamide (ASTA Medica). The drugs remained in the medium until cell harvest.

Xenograft experiments

A375 cells (8 × 106) were injected in the left and the right flank of immunodeficient mice (NOD.CB17-Prkdcscid/J). When tumors reached a suitable size, all animals were injected with O6BG (30 mg/kg i.p., dissolved in 1/3 PEG 400 and 2/3 PBS). Two hours later, vehicle or fotemustine (70 mg/kg i.p., dissolved in 1/4 ethanol and 3/4 PBS) was administered. Forty hours later, the mice were sacrificed and tumors were isolated, immediately frozen in liquid nitrogen, and stored at −80°C. For expression analysis, the tissue was disintegrated using a tissue lyser (Retsch) and RNA or protein was isolated as stated below.

Preparation of cell extracts and Western blot analysis

Whole-cell extracts and nuclear cell extracts were prepared as described (31). Antibodies used were: anti-PolH (ab17725, Abcam), anti-p53 (sc100, Santa Cruz Biotechnology), anti-phospho-p53Ser15 (9284, Cell Signaling Technology), anti-β-Actin (sc-47778, Santa Cruz Biotechnology), and anti-ERK2 (sc-154, Santa Cruz Biotechnology).

Preparation of RNA, end point PCR, and qRT-PCR

Total RNA was isolated using the RNA II Isolation Kit (Machery and Nagel) and cDNA synthesis was performed using the Verso cDNA Kit (Thermo Scientific). Endpoint PCR was performed using Red-Taq Ready Mix (Sigma-Aldrich) and quantitative real-time PCR (qRT-PCR) using the SensiMix SYBR Green & Fluorescein Kit (Bioline). The specific primers are depicted in Supplementary Table S1.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as described (32). PCR was performed using specific primers flanking the p53-binding site of polH (hPolH-ChIP-up: GAGCTCGAGAAATCTAGGCT, hPolH-ChIP low: AAATCTGGAAACC ATCAGGCT; (33) and as negative control, β-actin–specific primers (hActin-up: TCGCTGCCCAGGCACTC, hActin-low: GAGCGCGCAGTCAACGGA).

Overexpression and knockdown of PolH

For transient transfection, 1 × 106 cells were seeded per 10-cm dish. One day later, cells were transfected with 2 μg of pcDNA3.1 or pcDNA3.1-polH vector, by use of Effectene reagent (Qiagen). Knockdown of PolH was performed using the siRNA from Santa Cruz Biotechnology (sc-36289) and the Lipofectamine RNAiMAX Transfection Kit (Invitrogen).

Determination of apoptosis

For monitoring drug-induced apoptosis, ethanol-fixed cells were stained with propidium iodide as described (34). The sub-G1 fraction was determined by flow cytometry. Experiments were repeated at least three times, mean values ± SD are shown, and data were statistically analyzed using Student t test.

Immunofluorescence

Cells were seeded on coverslips. Following genotoxin exposure, cells were washed with PBS and fixed with 4% paraformaldehyde. After washing with PBS/0.2% Tween, a second fixation step was performed using 100% methanol, followed by additional washing steps with PBS/0.2% Tween. The antibodies used were anti-PolH (Abcam, ab 17725), anti-γH2AX (#05-164, Upstate), and as secondary antibody Alexa Fluor 488 (Ab') fragment of goat anti-rabbit IgG (Invitrogen, A11070). Nuclear staining was performed using TO-PRO3 iodide (Invitrogen). Slides were mounted in Vectashield medium (Vector Laboratories Inc.) and analyzed using a...
confocal laser scanning microscope (LSM 710, Zeiss) or the Metafer Scanning System (Metasystems). For removing non-DNA-bound PolH, cells were washed with cold PBS and then treated with 0.1% NP40 in PBS for 40 seconds on ice. After a quick PBS washing step, the cells were fixed and treated as described above. For EdU (5-ethynyl-2'-deoxyuridine) containing, cells were washed with PBS and then treated with 10 μmol/L EdU (Click it EdU Imaging Kit, C10338 Alexa Fluor 555, Invitrogen) for 1 hour. After a quick PBS washing step, the cells were fixed as described above. Before the nuclear staining, the 'Click it’ reaction was performed as proposed in the manufacturers’ instructions.

**Host cell reactivation assay**

To induce DNA damage, the firefly luciferase expression vector pGL2-con (Promega) was ex vivo exposed to different doses of fotemustine in Tris-EDTA buffer at room temperature for 12 hours. Thereafter, the plasmid was purified by phenol/chloroform extraction. Two-hundred nanograms of plasmids (180 ng of modified pGL2-con and 20 ng of the Renilla luciferase expression vector pRLEF-1α) were used for transfection (Effector Transfection Kit, Qiagen). Twenty-four hours after DNA transfection, the luciferase activities were measured using Dual-Glo luciferase assay. Reactivation of the pGL2-con plasmid was measured as activity of the firefly luciferase. Activity of the Renilla luciferase expressed by the cotransfected plasmid pRLEF-1α was used as control for transfection efficiency and subtracted.

**BrdUrd incorporation**

Cells were cultured in RPMI (10% FBS) and after exposure to fotemustine, bromodeoxyuridine (BrdUrd; 10 μmol/L) was added to the medium. One-hour later, the incorporation was analyzed by the BrdU Incorporation Kit (Roche Diagnostics). Experiments were repeated at least three times, mean values ± SD are shown, and data were statistically analyzed using Student t test.

**ICl repair measured by single-cell gel electrophoresis**

For the modified comet assay (35), cells were treated with 10 μg/ml fotemustine and, after the indicated time periods, trypsinized and washed with ice-cold PBS. Thereafter, cells were irradiated by 8 Gy. Alkaline cell lysis and electrophoresis was performed as described (36). The results are expressed as relative tail moment (%) = fluorescence intensity tail (%) × distance head center to tail end.

**MTT assay**

The metabolic activity of fotemustine-treated cells in comparison with untreated cells was determined by MTT assay. Seventy-two hours after exposure to the given anticancer drug, the medium was removed and 50 μL MTT solution (5 mg/mL in PBS) and 100 μL noncolored DMEM medium were added. After 3 to 4 hours of incubation, the medium was aspirated. The cells were lysed and the blue redox product was dissolved by adding 3 to 4 hours of incubation, the medium was aspirated. The cells were washed with PBS and then treated with 10 μmol/L EdU (Click it EdU Imaging Kit, C10338 Alexa Fluor 555, Invitrogen) for 1 hour. After a quick PBS washing step, the cells were fixed as described above. Before the nuclear staining, the 'Click it’ reaction was performed as proposed in the manufacturers’ instructions.

**Impact of p53 and replication arrest on fotemustine-induced PolH induction**

Upregulation of polH was previously reported for human fibroblasts upon treatment with ionizing radiation, with p53 being involved (33). Therefore, we analyzed the fotemustine-mediated induction of polH in melanoma cell lines proficient and deficient for p53 (Supplementary Fig. S1A). Induction of polH mRNA was only observed in p53-proficient melanoma cell lines (Supplementary Fig. S1B). We also observed induction of the canonical p53 targets p21, gadd45a, and fasR, substantiating the p53 wt status of the melanoma lines (Supplementary Fig. S1B). To further investigate the impact of p53 on polH induction, p53 was inhibited by Ptho, which has been shown to inhibit p53-dependent gene transcription (38). Pretreatment of D05 cells with 10 μg/ml Ptho completely abrogated the

**Results**

**Expression of PolH upon fotemustine exposure**

In melanoma cells, toxicity induced by chloroethylylating anticancer drugs like fotemustine depends on the p53 and MGMT status (23, 37). The melanoma cell line D05 is p53 wild-type (wt) and expresses MGMT, whereas the melanoma cell line D03 is deficient for both proteins (23). Therefore, D05 cells are highly resistant against fotemustine as compared with the melanoma cell line D03. Treatment with the p53 inhibitor pifithrin α (Ptho) or the MGMT inhibitor O6-benzylguanine (O6-BG) enhanced the cytotoxic potential of fotemustine in D05 cells (Fig. 1A). Combined treatment with the inhibitors further sensitized D05 cells to fotemustine nearly to the level observed in D03 cells (Fig. 1A). Similar results were observed using siRNA (data not shown). To further investigate the molecular mechanism underlying the protective effect of p53 on D05 cells upon treatment with fotemustine, MGMT was inhibited by O6-BG in all experiments to avoid repair of fotemustine-induced O6-CIG adducts.

To identify DNA repair genes that contribute to p53-mediated protection against chloroethylylating agents, qRT-PCR arrays were conducted, analyzing the expression of 180 DNA repair and DNA repair–associated genes. In this screening, we observed the induction of the PolH in the p53 wt melanoma cell line D05 after fotemustine treatment (data not shown). To verify the induction of PolH upon fotemustine, the time-dependent expression of polH mRNA was examined by end point PCR (Fig. 1B) and qRT-PCR (Fig. 1C). An enhanced expression of polH mRNA was detectable 8 hours after treatment and was constantly increasing during the 24-hour post-incubation period. Induction of polH mRNA was accompanied by an increase in the level of PolH protein (Fig. 1D). The enhanced expression of PolH was observed between 16 and 32 hours after exposure and was preceded by nuclear accumulation of p53 (Fig. 1D). To clarify whether the induction of polH mRNA and protein is the result of promoter activation, cells were treated with the transcription-blocking agent actinomycin D (ActD). Treatment with the inhibitor only slightly reduced the basal level of polH mRNA, but completely prevented the increase of polH mRNA upon fotemustine, suggesting the induction of polH mRNA to be dependent on RNA de novo synthesis (Fig. 1E).
induction of PolH upon fotemustine exposure, both on mRNA (Fig. 2A) and on protein level (Fig. 2B), supporting the notion that PolH is transcriptionally regulated by p53. Finally, ChIP experiments showed in vivo binding of p53 protein to the p53-binding site of the polH promoter in D05 cells exposed to fotemustine (Fig. 2C).

An intriguing question is whether induction of PolH is triggered by fotemustine-induced ICL, by the formation of DNA repair intermediates like DSBs, or DNA replication arrest. To clarify this, the activation of p53 was analyzed via immunodetection of p53Ser15 (Fig. 2D), the induction of PolH by qRT-PCR (Fig. 2E), replication blockage by BrdUrd assay (Fig. 2F) and the formation of DSBs via γH2AX foci (Fig. 2G). To inhibit replication, we used hydroxyurea (HU), a typical DNA replication blocking agent. Upon exposure of D05 cells to fotemustine (Fig. 2C).

Upon fotemustine, p53 activation and polH induction was more pronounced in the HU-exposed cells, reaching its maximum already 2 hours after treatment (Fig. 2D and E). Upon HU exposure, no DSBs were observed 2 to 8 hours after treatment although a strong pan-staining of γH2AX was detected (data not shown), indicating the replication arrest triggered activation of the DNA damage response (DDR) and, concomitantly, induced polH. However, following fotemustine, which only modestly induced replication arrest in the dose range used, the formation of DSBs seems to play a predominant role in activating the DDR and thereby the induction of polH. As neither p53 activation nor polH induction was observed at early time points following fotemustine, we conclude that the monoadducts formed by the agent do not activate the DDR and do not induce polH.

**Induction of PolH in tumor cells, melanoma xenografts, and nontransformed cells**

To analyze whether the induction of PolH is restricted to melanoma cells or is a common response of tumor cells to crosslinking anticancer drugs, different cell systems were analyzed concerning the inducibility of PolH. In U87 glioma cells, a time-dependent induction of polH mRNA (Fig. 3A and B) and PolH protein (Fig. 3C) was observed following treatment with CCNU (used for glioma therapy). Similar to melanoma cells, the induction of PolH was abrogated when cells were...
pretreated with Pthα (Fig. 3D). To further analyze the p53 dependency of polH induction in glioma cells, we used a panel of glioma cell lines well characterized to the p53 status (30). By using these lines, induction of polH mRNA was only observed in the p53-proficient glioma cell line LN229, but not the p53-deficient (LN308) or p53-mutated (T98G, LN18, LN319, U138) cells (Fig. 3E), indicating that p53 mutations impairing p53 activity prevent polH upregulation following ICL drug treatment.

Induction of polH mRNA was also observed in breast cancer cells (MCF7) and lymphoblastoid cells (TK6) upon treatment with mafosfamide (Fig. 3F). As fotemustine, CCNU, and MAF all induce ICLs, the data support the view that induction of PolH is a common response to ICL-inducing anticancer drugs.

To study whether PolH becomes upregulated in cells grown in a host in vivo, A375 melanoma cells wt for p53 were injected into immunodeficient mice. Upon tumor formation, mice were either mock-treated or treated with fotemustine (70 mg/kg, 81 62 4 Time (h))

Figure 2. p53-dependent and fotemustine-triggered induction of PolH in melanoma cells. A and B, D05 cells were exposed to 10 μg/mL fotemustine (FM) in the presence or absence of 10 μmol/L Pthα. Expression of polH mRNA was analyzed by end point PCR (A) and expression of PolH protein was analyzed by Western blot analysis using nuclear protein extracts (B). C, ChIP analysis was performed in untreated D05 cells and cells exposed to 10 μg/mL fotemustine for 16 hours. One percent of cell extracts from each of the samples was taken as input. Protein-DNA complexes were immunoprecipitated with anti-p53, anti-ERK1/2, or without antibody and end point PCR was performed using primers flanking the p53-responsive element within the polH promoter or primers specific for the β-actin coding sequence. D–G, D05 cells were exposed to 10 μg/mL fotemustine or 1 mmol/L HU for indicated times. D and E, phosphorylation of p53Ser15 was analyzed by Western blot analysis (D) and expression of polH mRNA by qRT-PCR (E). F, DNA replication was measured by BrdUrd incorporation. G, formation of γH2AX foci was analyzed by microscopy. Eight-hundred cells were scored for each time point and the percentage of cells showing >3 γH2AX foci are presented.
i.p.). Tumors were removed 40 hours thereafter and the expression of PolH was measured on mRNA and protein level. As indicated by end point PCR (Supplementary Fig. S1C) and qRT-PCR (Fig. 4A), the expression of polH mRNA was higher in all samples obtained from fotemustine-treated animals compared with samples obtained from untreated mice, which were randomly crosspaired. An enhanced expression of PolH and p53 was also observed on protein level in the melanoma xenografts following treatment of mice with fotemustine (Fig. 4B).

PolH induction is not a tumor-specific trait; it was also found in nontransformed cells like human diploid fibroblasts (VH10tert; Fig. 4C) and in replicating (CD3/CD28 stimulated) PBLCs obtained from healthy donors (Fig. 4D and E) following exposure to fotemustine. Of note, polH induction was not observed in nonstimulated PBLCs, which are in G0 (>95%; Fig. 4E). The lack of induction of polH in nonproliferating PBLCs, along with lack of p53 activation (Fig. 4F), supports the view that not the primary DNA damage, but replication-associated lesions (DSB) trigger the response.

**Impact of PolH on the sensitivity to chloroethylating anticancer drugs**

PolH was related to both, resistance and sensitivity to genotoxic stress. Thus, PolH deficiency renders human fibroblasts sensitive to UV light, but resistant to ionizing radiation (33). To analyze the impact of PolH on chloroethylating anticancer drug-induced toxicity, PolH was downregulated via siRNA or ectopically overexpressed. The determination of cell viability via the MTT assay showed a protective effect of PolH against several crosslink inducing anticancer drugs. In detail, PolH knockdown sensitized melanoma cells to fotemustine, glioma cells to CCNU, and breast cancer cells to MAF (Fig. 5A), drugs used in the treatment of the corresponding types of tumors. To further analyze the impact of PolH on cell death, the sub-G1 fraction was determined. Also in this assay, knockdown of PolH had a strong impact on fotemustine-induced toxicity in D05 cells and clearly enhanced cell death (Fig. 5B). The efficiency of PolH knockdown was verified in parallel experiments by Western blot analysis (Fig. 5B, left). To assess the impact of PolH induction on fotemustine resistance, p53-deficient D03 melanoma cells, showing no induction of PolH upon fotemustine exposure (Supplementary Fig. S1B), were transfected with a PolH expression vector (Fig. 5C, left plot for efficiency of PolH expression) and exposed to fotemustine. Overexpression of PolH clearly protected the cells against fotemustine-induced cytotoxicity (Fig. 5C, right panel).

**Impact of PolH on the repair of fotemustine-induced DNA damage**

Next, we analyzed whether PolH is involved in the repair of fotemustine-induced DNA damage. Therefore, host...
reactivation assays were performed, transfecting ex vivo fotemustine-exposed pGL2-con plasmids into D05 cells. After dose-finding experiments (Supplementary Fig. S1F), pGL2-con plasmids alkylated with 5 and 25 μg/mL fotemustine were transfected either in polH siRNA-transfected, nonsilencing (ns) siRNA-transfected or nontransfected cells and reactivation of the plasmid was determined 24 hours later. The data clearly show that knockdown of PolH negatively affects the ability of cells to reactivate the plasmid (Fig. 6A), indicating its involvement in the repair of chloroethylating agent-induced DNA damage. Besides performing host cell reactivation assays, we also analyzed the formation and repair of fotemustine-induced DNA damage in vivo. Therefore, the modified alkaline comet assay was used to monitor the repair of DNA crosslinks. The data show that PolH is not involved in the early step of ICL repair as its knockdown does not affect ICL formation or its resolution (Fig. 6B).

ICLs represent a severe block of DNA replication, causing DSB at blocked replication forks. To analyze the impact of PolH on replication and DSB formation, replication was analyzed by BrdUrd assay and DSB formation by γH2AX foci. Knockdown of PolH clearly extended the fotemustine-induced block to replication (Fig. 6C) and, in parallel, increased the amount of γH2AX foci (Fig. 6D). Whereas the amount of γH2AX foci per cell was <3 in untreated cells, fotemustine (10 μg/mL) induced approximately 40 γH2AX foci per cell 24 hours after exposure (Fig. 6D). Upon siRNA-mediated knockdown of PolH, the number of γH2AX foci was significantly enhanced 48 and 72 hours after fotemustine in comparison with ns-siRNA–transfected cells (Fig. 6D). We should note that only a subfraction of cells showed γH2AX foci. Therefore, only cells showing >3 foci per cell were included in the quantification. Also, the percentage of cells showing >3 γH2AX foci upon fotemustine exposure was higher in PolH siRNA-transfected cells (Supplementary Fig. S1G). The finding that only a subpopulation of cells showed...
γH2AX foci indicates that its formation is associated with replication. This is supported by EdU labeling experiments. Formation of γH2AX foci was observed predominantly in replicating cells (Supplementary Fig. S2A); 24 hours after fotemustine exposure, more than 85% of cells positive for EdU showed γH2AX foci (Supplementary Fig. S2B), indicating that DSB formation following fotemustine is restricted to S-phase cells.

To further prove that PolH is involved in the replication-associated repair of chloroethylating agent-induced DNA damage, notably ICL, the cellular localization of PolH was analyzed. Enhanced nuclear expression of PolH was observed in fotemustine-exposed cells (Supplementary Fig. S2C). In experiments, in which strong washing conditions were applied to remove non DNA-bound proteins (39), PolH was not detectable in untreated cells (Fig. 6E). In fotemustine-treated cells, however, a strong staining of PolH was observed in 20% to 30% of the cells. Parallel detection of EdU incorporation showed that binding of PolH to the DNA in fotemustine-treated cells occurs predominantly (>80%) in replicating (S-phase) cells (Fig. 6E and Supplementary Fig. S2B).

Discussion

In glioblastoma and melanoma cells, p53 is one of the major factors influencing toxicity of anticancer drugs. In case of the chloroethylating agents, ACNU (22) and fotemustine (25), this protective effect was related to the induction of the nucleotide excision repair genes xpc and ddb2, which are robustly regulated by p53. To identify additional factors involved in p53-mediated resistance to ICL-inducing agents, we analyzed the p53-mediated regulation of all known DNA repair genes and identified the PolH as an important p53-regulated factor involved in the cellular protection against fotemustine and other ICL-inducing anticancer drugs.

Induction of PolH by fotemustine was found in melanoma cell lines, normal fibroblasts, and proliferating PBLCs on the level of mRNA and protein. Furthermore, induction of PolH was observed in glioma cells upon exposure to CCNU used in glioma therapy and in breast cancer cells upon exposure to MAF, a derivative of the crosslink inducing anticancer drug cyclophosphamide used in breast cancer therapy. PolH induction was mediated by p53 as substantiated by ChIP
experiments and experiments using the p53 inhibitor Ptho.

This notion is in line with a previous report showing p53-dependent induction of PolH by IR and camptothecin (33). The observation of PolH induction in tumor cell lines grown in vitro and as xenografts, and in PBLCs upon exposure to various crosslink-inducing agents clearly hints to an important and widely spread biologic function of PolH upregulation. PolH seems to play different roles in cellular toxicity according to the type of DNA damage. Thus, knockdown of PolH on the one hand enhanced the sensitivity to UV light and, on the other, enhanced cellular resistance to IR (33). To analyze the impact of PolH on anticancer drug–induced cytotoxicity, PolH was downregulated using siRNA. Downregulation of PolH clearly sensitized melanoma cells to fotemustine, glioma cells to CCNU, and MCF7 cells to MAF, indicating that PolH is a general player involved in the protection of cancer cells against crosslink-inducing agents. Furthermore, ectopic expression of PolH in D03 melanoma cells rendered them more resistant to fotemustine, supporting the notion that PolH induction contributes to ICL drug resistance.

Our data suggest that PolH represents a potential marker of cancer cell resistance. How is PolH expressed in tumors? As data are scarce, we initially analyzed the expression of PolH in malignant glioma cell lines (Supplementary Fig. S3A) and in human breast cancer and corresponding normal tissue (Supplementary Fig. S3B and S3C). The results showed clear variations in the PolH expression, which was overall slightly higher in cancer than in the normal tissue, indicating that PolH might be upregulated during tumor development. However, more studies concerning the expression of PolH in tumors are needed to clarify this point.

We also analyzed whether PolH is directly involved in the repair of chloroethylating agent–derived DNA adducts. Fotemustine chloroethylates the O6-position of guanine, forming O6-ClG, which thereafter undergoes intramolecular rearrangement to form an ICL (5). Whereas O6-CIG is repaired by MGMT

Involvement of Polymerase η in Anticancer Drug Resistance

Figure 6. Impact of PolH on the repair of fotemustine-triggered DNA damage. A–D, D03 cells were either nontransfected (nt) or transfected with ns-siRNA or PolH-specific siRNA. A, host cell reactivation assay. Cells were transfected 24 hours later with the plasmid pgL2-con, which was ex vivo exposed to 5 or 25 μg/mL fotemustine (FM) and with the plasmid pRLEF-1α. Sixteen hours later, the cells were harvested, protein extracts isolated, and subjected to the dual luciferase assay. B, ICL repair was measured by modified comet assay at 24 and 48 hours after fotemustine (10 μg/mL) treatment. C, DNA replication was measured by BrdUrd incorporation assay 24, 48, and 72 hours after fotemustine (10 μg/mL) treatment. D, formation of γH2AX foci was analyzed by confocal laser scanning microscopy at 24, 48, and 72 hours after fotemustine (10 μg/mL) treatment. Fifty cells (showing >3 foci) were scored for each time point. A–D, data are the mean of three independent experiments ±SD. **P < 0.01; ***P < 0.001. E, binding of PolH to fotemustine-damaged DNA was determined using immunofluorescence and confocal laser scanning microscopy 16 hours after exposure to 10 μg/mL fotemustine. Nuclear staining was performed using TO-PRO3 and detection of replicating cells was analyzed by EdU incorporation.
the repair of ICL is complex, involving multiple repair mechanisms (1). Also to consider, ICL repair differs between replicating and nonreplicating cells. In nonreplicating cells, the repair depends on nucleotide excision repair (NER)-mediated recognition of the crosslink and dual NER-mediated incision at positions flanking the crosslinked DNA. After flipping the excised DNA fragment out of the helix, resynthesis of DNA occurs and a second cycle of NER finally removes the flipped-out crosslink (1). Whether resynthesis of DNA is accomplished by replicative or translesion polymerases is unclear. In replicating cells, when the crosslink encounters the replication fork, NER, TLS, and HR are implicated in the repair (1).

To analyze the role of PolH in the repair of fotemustine-induced DNA adducts, host reactivation assays were performed. Upon knockdown of PolH, a clear decrease in plasmid reactivation was observed, indicating that PolH is involved in the repair of fotemustine-induced DNA damage. An intriguing question is whether PolH is involved in the repair of the monoadducts or ICL. In vitro, conversion of a monoadduct to a crosslink occurs within a period of 3 hours (5). In cells upon exposure to chloroethylating drugs, the formation of ICL was finished within 8 hours (41). As ex vivo modification of the plasmid was performed in our experiments for 12 hours, O6-methylguanine should not be present anymore, supporting the notion that PolH is involved in the repair of ICL. This is in line with data showing repair of mitomycin C-induced ICL using in vivo reactivation assays. In these experiments, using xeroderma pigmentosum patient–derived XPV cells, partial requirement for PolH was observed (42). Moreover, XPV cells are hypersensitive to cisplatin (43, 44), and PolH was associated with processing of psoralen-induced crosslinks (45).

We also performed modified alkaline comet assays to measure the repair of ICL in vivo. No difference was observed in the formation and initial cleavage of ICL, indicating that PolH is not involved in the first steps of ICL repair, namely recognition and incision. However, a prolonged replication block and an enhanced persistence of γH2AX foci were observed, indicating that the efficient execution of later steps of ICL repair is reduced in PolH-compromised cells. Besides its role in TLS, PolH displays an important function in mitotic gene conversion and homologous recombination (46). Furthermore, PolH possesses a dual role at stalled replication forks, promoting TLS and reinitiating DNA synthesis during HR by primer extension of D-loop structures (47). More recently it was shown that PolH provides a supportive role for PolD during normal replication (48). Therefore, the question arises whether the function of PolH in ICL repair following anticancer drug is associated with replication events. To address this, we analyzed whether PolH can directly interact with fotemustine-damaged DNA in vivo and whether this reaction is associated with replication. Upon removal of non-DNA–bound PolH, the remaining DNA-bound PolH was only detected in fotemustine-treated, but not in unexposed cells. Parallel detection of EdU incorporation showed that binding of PolH to the DNA of fotemustine-exposed cells occurs predominantly in replicating cells (>80% of PolH-positive cells were at the same time positive for EdU incorporation), indicating a role for PolH in replication-associated repair of ICL. This finding is in contrast to data showing that DNA damage–induced PolH recruitment takes place independently of the cell-cycle phase (49). This, however, was observed after treatment with UV light, which induces intrastrand crosslinks that are repaired independently of replication, whereas ICL are more severe lesions that are repaired predominantly within the S-phase, which is supported by a large fraction of the cells showing γH2AX foci in the S-phase upon fotemustine treatment.

Upregulation of PolH following anticancer drug treatment was not only observed in malignant cells, but also in fibroblasts and PBLCs isolated from healthy individuals. As PolH plays a protective role, the data suggest that PolH may also protect the normal tissue from the therapeutic side effects of ICL-inducing anticancer drugs. We should also note another interesting side effect. As PolH, a low fidelity TLS polymerase, is able to insert wrong nucleotides opposite to the lesion in the DNA (26), its activity is error-prone, leading to the formation of mutations. These might be a source of therapy-induced secondary tumors, which is indeed a serious deleterious side effect of ICL-inducing drugs such as nitrogen mustards and nitrosoureas (50).

In summary, we show for the first time upregulation of PolH upon exposure of cells to crosslink-inducing anticancer drugs. The process is dependent on p53 and was observed in melanoma, glioma, and breast cancer cell lines. It was also observed in a mouse melanoma xenograft model in the absence of systemic toxicity, indicating that it occurs in a relevant dose range. Furthermore, we show that the elevated expression of PolH enhances the resistance of cancer cells to crosslinking anticancer drugs, including chloroethylating agents and cyclophosphamide derivatives, resulting from a participation of PolH in the repair of DNA crosslinks.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.T. Tomicic, B. Kaina, M. Christmann
Development of methodology: D. Aasland
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Meise, C. Barckhausen, M. Christmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.T. Tomicic, R. Meise, B. Kaina, M. Christmann
Writing, review, and/or revision of the manuscript: M.T. Tomicic, B. Kaina, M. Christmann
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.C. Naumann, B. Kaina
Study supervision: M. Christmann

Acknowledgments
The authors thank Birgit Rasenberger for excellent technical assistance.

Grant Support
The work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG CH 665/2-2).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 4, 2014; revised June 7, 2014; accepted June 30, 2014; published OnlineFirst August 14, 2014.
Involvement of Polymerase η in Anticancer Drug Resistance

References


Translesion Polymerase η Is Upregulated by Cancer Therapeutics and Confers Anticancer Drug Resistance

Maja T. Tomicic, Dorthe Aasland, Steffen C. Naumann, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0953

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/08/16/0008-5472.CAN-14-0953.DC1

Cited articles
This article cites 50 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/19/5585.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/74/19/5585.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.