Lack of correlation between repair of DNA interstrand cross-links and hypersensitivity of hamster cells towards mitomycin C and cisplatin

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Abstract The ability to repair DNA interstrand cross-links may be an important factor contributing to mitomycin C (MMC) and cisplatin cytotoxicities. We have assessed the repair of interstrand cross-links induced by MMC in two MMC-hypersensitive hamster cell mutants and their resistant parental cell line. Using a gene-specific repair assay, we found no evidence for repair of MMC cross-links in either parental or mutant cells, suggesting that persistence of DNA interstrand cross-links is not responsible for the differential toxicity of MMC towards hypersensitive cells. Repair of cisplatin-induced interstrand cross-links was efficient in resistant as well as in mutant cells. Therefore we concluded that a defect in excision repair of interstrand cross-links was not responsible for the cytotoxic effects of MMC and cisplatin in these hypersensitive mutants.

Key words: DNA repair; Interstrand cross-link; Mitomycin C; Cisplatin

1. Introduction

Interstrand DNA cross-links represent an important class of chemical damage to DNA, since they prevent DNA strand separation and thus can constitute complete blocks to DNA replication and transcription. Since cross-linking of DNA has been considered to be responsible for cytotoxicity, a number of bifunctional agents such as cisplatin, mitomycin C, and nitrogen mustard have been used extensively in cancer chemotherapy, as single agents or in combination in the treatment of a wide variety of malignant tumors [1].

Cisplatin and mitomycin C (MMC) are both structurally and chemically distinct. Cisplatin preferentially forms lesions at the N-7 position of guanine [2], whereas MMC mainly alkylates the N-2 position of guanine, upon reductive activation cascade [3]. Cisplatin can generate intra- and interstrand cross-links, both of which have been implicated in cytotoxicity [4,5]. In contrast, the toxicity of MMC has been mainly correlated with the formation of interstrand cross-links [3]. Although most of the chemical lesions produced by these anticancer drugs are known, the molecular basis of their antitumor activity is still to be understood. DNA repair efficiency and inhibition of DNA synthesis [6], as well as cell-cycle checkpoint responses and induction of cell death by apoptosis [7], have been considered the critical steps in toxicity.

To elucidate the mechanisms of the mammalian cell defence against cross-linking agents, mutants specifically sensitive to MMC have been isolated in rodent cells [8]. The genetic and biochemical complexity of these processes is reflected by the existence of at least eight complementation groups identified among rodent cell mutants defective in this response [9,10]. V-H4 and V-C8 cell mutants are representatives of two different complementation groups and were isolated from V79 Chinese hamster cells [11,12].

The V-H4 mutant cell line shows many typical characteristics of cells derived from Fanconi anemia (FA) patients [11,13]. V-H4 cells exhibit increased sensitivity towards cross-linking agents such as MMC, cisplatin, and diepoxybutane (DEB), but are not sensitive to UV light and X-rays [12]. The V-C8 mutant cell line also shows increased sensitivity towards cross-linking agents, such as MMC, cisplatin, and DEB, but is only slightly sensitive to UV and X-rays [12]. This combined analysis of the response of V-H4 and V-C8 cells to a panel of cytotoxic agents suggests that the defective proteins in these cells are involved in cellular responses to cross-linking agents such as MMC and cisplatin.

To assess whether a repair defect is involved in the specific hypersensitivity of V-H4 and/or V-C8 cells to bifunctional agents, we have studied the formation and repair of MMC- and cisplatin-induced DNA interstrand cross-links in these mutants and their parental cell line V79. Interstrand cross-links were detected at the level of the active and multi-copies ribosomal RNA gene, using a gene-specific repair assay [14,15].

2. Materials and methods

2.1. Cells and culture conditions

The MMC-sensitive mutants V-H4 and V-C8 derived from Chinese hamster V79 cells have been described previously [8,11,12]. V79, V-H4 and V-C8 cell lines were routinely grown in monolayer in Ham's F-10 medium (Gibco) supplemented with 15% newborn calf serum (Gibco), penicillin (100 U/ml), and streptomycin (0.1 μg/ml). The Chinese hamster AA8 cell line and its derived mutant UV4 from UV complementation group 1 were provided by Dr. D. Bush and have been described previously [16]. AA8 and UV4 were routinely grown in monolayer in αMEM medium (Gibco) supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/ml), and streptomycin (0.1 μg/ml). All incubations were at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Cell treatments

For all experiments in this study, cells were in exponential growth phase at the time of DNA damage. Cells were treated with MMC (Sigma-Aldrich) for 1 h, or with cisplatin (Sigma-Aldrich) for 5 h, at the indicated concentrations at 37°C. After drug exposure, cells were washed with phosphate-buffered saline (PBS) and then incubated in fresh medium. In each case, control and treated cells were handled in the same way, with the only difference being omission of the drug treatment.

2.3. Cytotoxicity assay

Sensitivity to MMC was assessed in a colony-forming growth assay. Cells were plated at various dilutions 1 day prior to treatment to allow attachment. The attached cells were then treated with different concentrations of MMC for 1 h at 37°C. After drug treatment, cells

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were rinsed twice in PBS, and fresh medium was added. The cells were allowed to grow for 8 days and were then fixed and stained in methylene blue solution (0.25%) and counted. The colony-forming efficiencies of the V79, V-H4 and V-C8 cells were routinely 80%, 65% and 50%, respectively. Drug treatments were done in triplicate at each concentration and the cell survival was expressed relative to the number of colonies obtained without drug.

2.4. Gene-specific repair assay

A denaturation-renaturation gel electrophoresis method was used to detect DNA interstrand cross-links induced by MMC or cisplatin [14,15]. Cellular DNA was prelabeled by allowing cells to grow for 64 h at 37°C in [*methyl*-^{3}H]thymidine at 0.3 μCi/ml and 10 μM thymidine. Following this procedure, the cells were subcultured and incubated in label-free medium for 24 h. Cells were then treated with 30 μM MMC for 1 h or with 200 μM cisplatin for 5 h. After drug exposure, cells were washed twice with PBS, and lysed either immediately or after repair incubation in a solution of 0.5 M Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS and 0.5 mg/ml proteinase K for 16 h at 37°C. Cells used for repair analysis were incubated for 24 or 48 h at 37°C in medium supplemented with 10 μM bromodeoxyuridine and 1 μM fluorodeoxyuridine to density label the DNA replicated after MMC or cisplatin treatment. High molecular weight DNA was isolated and purified by a salt lysis method [17], and then restricted with *Hin*dIII (Biolabs) restriction endonuclease (2 U/μg DNA) and treated with 10 μg/ml RNase A for 3–4 h. Samples were centrifuged to equilibrium in a neutral CsCl gradient containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE). Fractions of parental and replicated DNA were then pooled separately, dialyzed and resuspended in TE.

After treatment in 50 mM NaOH for 20 min at 37°C, cross-linked DNA was resolved from single-stranded DNA on a 0.6% agarose gel at 30 V for 16–18 h. The DNA was transferred after electrophoresis to Sureblot nylon membrane (Oncor, Appligene) in 1 M NaOH. The membranes were prehybridized in Hybrisol I (Oncor), hybridized with ^{32}P-labeled DNA probe and then quantified using a PhosphorImager (Molecular Dynamics Inc.) and ImageQuant software (Molecular Dynamics Inc.). The 1.4-kb human 28S genomic probe (pA_{BB}) for the ribosomal RNA gene is homologous to the hamster sequence [18] and was used to detect a 20-kb *Hin*dIII fragment containing the 5.8S and 28S rRNA sequences in hamster cells. The degree of cross-linking was calculated from the ratio of double-stranded to total DNA in each lane. The Poisson distribution was applied to calculate the average number of cross-links (= −Ln (fraction of molecules free of cross-links)). Data were generated from three to five biological experiments with three to six gels per experiment.

3. Results

3.1. Cell survival after MMC treatment

To confirm the hypersensitivity of the mutant cell lines towards cross-linking agents in our repair assay conditions, we determined the MMC cytotoxicity in these cell lines. The survival curves of parental V79 and mutant V-H4 and V-C8 cells treated for 1 h with increasing concentrations of MMC are presented in Fig. 1. The IC_{90} values (dose required to reduce cell survival to 10%) of V79, V-H4 and V-C8 are 15 μM, 0.45 μM and 0.15 μM respectively (Fig. 1). Thus, we confirmed that the mutant cells are 30-fold (V-H4) and 100-fold (V-C8) more sensitive to MMC treatment than the parental cell line V79. Cisplatin cytotoxicity was also tested in our repair assay conditions: V-H4 cells are 10-fold and V-C8 cells 40-fold more sensitive to cisplatin treatment than V79 (data not shown).

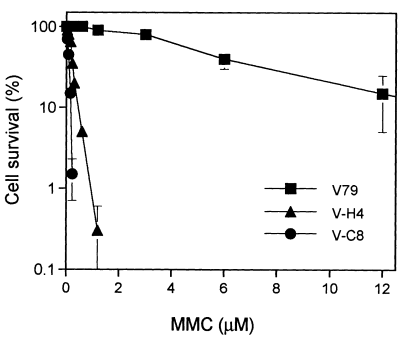

Fig. 1. Clonogenic assay following MMC treatment. The percent cell survival of the parental V79 cells and its mutants V-H4 and V-C8 is shown as a function of MMC dose. The data are derived from two independent experiments done in triplicate for each MMC concentration and are presented as mean values ± S.E.

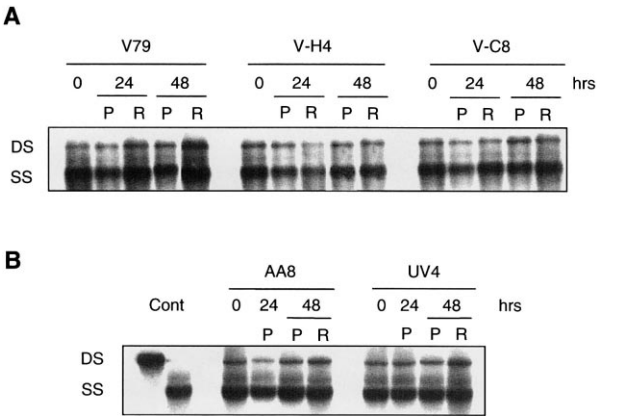

Fig. 2. Formation and repair of MMC-induced interstrand cross-links in the rRNA gene of hamster cells. A: Representative autoradiogram of Southern blot of *Hin*dIII genomic fragments, isolated from V79, V-H4 and V-C8 cell lines treated with 30 μM MMC for 1 h, and hybridized with ^{32}P-labeled probe for the rRNA region. B: Representative autoradiogram of Southern blot of *Hin*dIII genomic fragments, isolated from AA8 and UV4 cell lines treated with 30 μM MMC for 1 h, and hybridized with ^{32}P-labeled probe for the rRNA region. Cross-link detection in parental DNA (P) and newly replicated DNA (R) is shown following incubation at 0, 24, and 48 h with denaturation by NaOH. DS, double-stranded DNA. SS, single-stranded DNA. Cont, non-damaged DNA. Data were generated from five biological experiments with four to six gels per assay. The degree of cross-linking was calculated from the ratio of double-stranded to total DNA in each lane, using a PhosphoImager and ImageQuant software (Molecular Dynamics Inc.).

3.2. Formation and repair of DNA interstrand cross-links induced by MMC

To assess whether a repair defect is involved in the hypersensitivity of V-H4 and/or V-C8 cells to MMC, we examined the removal of MMC-induced interstrand cross-links from the rRNA gene in V79, V-H4 and V-C8 cells. The rRNA gene high copy number is transcribed at very high rates in exponentially growing cells. Interstrand cross-links were detected by a denaturation-renaturation method, after Southern transfer and hybridization, as described in Section 2.

To compare the repair characteristics of the parental and mutant cell lines, cells were treated with equimolar concentrations of drug (30 μM MMC) for 1 h. These concentrations of MMC are comparable to those achieved in plasma of humans (10 μM) [19]. Since we measured significant amounts of DNA synthesis during the incubation following MMC treatment, the subsequent gene-specific repair assays were done separately on parental DNA (P) or newly replicated DNA (R).

A representative autoradiogram, Fig. 2A, shows the formation ($t = 0$ h) and the processing ($t = 24$ h and 48 h) of MMC-

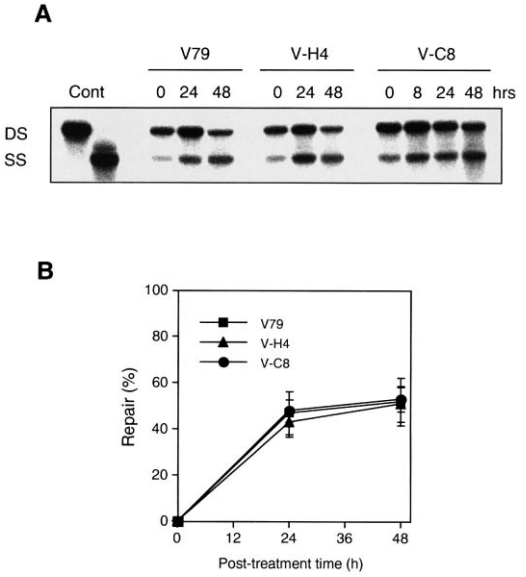

Fig. 3. Formation and repair of cisplatin-induced interstrand cross-links in the rRNA gene of hamster cells. A: Representative autoradiogram of Southern blot of *Hin*dIII genomic fragments, isolated from V79, V-H4 and V-C8 cell lines treated with 200 μM cisplatin for 5 h, and hybridized with ^{32}P-labeled probe for the rRNA region. Cross-link detection in parental DNA is shown following incubation at 0, 24, and 48 h with denaturation by NaOH. DS, double-stranded DNA. SS, single-stranded DNA. Cont, non-damaged DNA. B: Data were derived from quantitation of Southern blots using a PhosphorImager and ImageQuant software (Molecular Dynamics) and are presented as mean values ± S.E. from three biological experiments, with two to four sets of gel electrophoresis for each experiment. Values were determined from scans of double-stranded DNA versus total (single- plus double-stranded) DNA for each time point.

induced interstrand cross-links within a 20-kb fragment of the rRNA gene of the parental DNA (P) and the newly replicated DNA (R) of V79, V-H4 and V-C8 cells. The treatment induced a comparable number of cross-links per fragment (0.1 cross-link/10 kb at time point $t = 0$ h) in the three cell lines (Fig. 2A), suggesting that the bioactivation of MMC is similar in resistant and sensitive cell lines. Surprisingly, no significant removal of MMC interstrand cross-links from the parental DNA (P) of the three cell lines was observed during the subsequent incubation, since the amount of interstrand cross-links remained constant at time points 0, 24 and 48 h (0.09–1.2 cross-link/10 kb in parental DNA) (Fig. 2A).

Moreover, the MMC-induced cross-links originally formed in parental DNA (P) were also associated with newly replicated DNA (R) at time points 24 h and 48 h (Fig. 2A). The presence of DNA interstrand cross-links in the replicated DNA after MMC treatment might be a consequence of a recombinational mechanism occurring during replication past cross-links, or a conversion of monofunctional MMC adducts to interstrand cross-links during the repair kinetics. In order to test these hypotheses, we examined the removal of interstrand cross-links induced by MMC from the rRNA gene in a repair-proficient hamster cell line AA8 and its UV-sensitive repair-deficient mutant UV4. UV4 cells belong to complementation group 1 and cannot excise bulky lesions from DNA. AA8 and UV4 cell lines were thus treated with 30 μM MMC for 1 h and interstrand cross-links were detected by denaturation-renaturation assay. As shown previously for V79, V-H4 and V-C8 cells, there was no removal of MMC-induced interstrand cross-links from the parental DNA (P) of AA8 and UV4 cells (Fig. 2B). Furthermore, cross-links were also associated with newly replicated DNA (R) at time points 24 h and 48 h in these cells (Fig. 2B). Addition of the amount of interstrand cross-links in parental (P) plus replicated (R) DNA at time point 48 h displayed a higher quantity of cross-links (0.2 cross-link/10 kb) than the initial amount of cross-links induced at time point 0 h (0.1 cross-link/10 kb) in the parental DNA (P) after the MMC treatment in repair-deficient UV4 cells (Fig. 2B). Since these cells cannot excise MMC adducts, these findings demonstrate that there is probably a conversion of MMC-induced monofunctional adducts to interstrand cross-links during the post-treatment incubation.

Finally, MMC cross-link repair efficiencies could not be quantitatively compared between resistant and hypersensitive cell lines, since we measured a combination of formation and removal of MMC interstrand cross-links during the repair kinetics.

3.3. Formation and repair of DNA interstrand cross-links induced by cisplatin

To be able to compare the repair characteristics of both mutants versus the parental cell line, we then studied the removal of interstrand cross-links induced by a different cross-linking agent, from the rRNA gene of V79, V-H4 and V-C8 cells. Cells were treated with 200 μM cisplatin for 5 h, when cisplatin concentrations achieved in plasma of humans varies from 10 to 60 μM. Since none of the cell lines demonstrated significant replication within the time of the experiment, we only used the parental DNA in subsequent repair assays.

A representative autoradiogram, Fig. 3A, shows the formation ($t = 0$ h) and the removal ($t = 24$ h and 48 h) of cisplatin-induced cross-links within a 20-kb fragment of the rRNA of the parental DNA of V79, V-H4 and V-C8 cells. The treatment induced similar numbers of interstrand cross-links (about 0.8 cross-link/10 kb fragment) in the three cell lines (Fig. 3A) and the removal of these cross-links was effective within the three cell lines (Fig. 3A). Data from the cisplatin interstrand cross-links experiments are compiled in Fig. 3B: about 40–45% of the cross-links were repaired at $t = 24$ h and 50–55% at $t = 48$ h (Fig. 3A,B). The extent of cisplatin cross-link removal was also quantified once after 4 and 8 h of repair incubation. We found 16–20% repair within 4 h after exposure to the drug and 28–35% repair within 8 h for the three cell lines (data not shown). Unlike MMC-induced cross-links, cisplatin-induced cross-links were significantly removed from the rRNA sequence during the repair kinetics. However, there was no significant difference between resistant and sensitive cell lines regarding the repair of the cisplatin-induced interstrand cross-links, indicating that V-H4 and V-C8 cells are not deficient in the excision repair of these lesions.

4. Discussion

There are many mechanisms by which hypersensitivity to bifunctional agents can be achieved in cells. The ability to remove DNA interstrand cross-links may be an important factor contributing to MMC and cisplatin sensitivities. In this report, we have compared the cross-link formation and removal characteristics in a defined gene sequence of MMC-

hypersensitive V-H4 and V-C8 cell mutants with those of the resistant parental V79 cell line. Using a gene-specific detection assay, we measured equivalent amounts of MMC-induced DNA interstrand cross-links at the level of the rRNA gene in V79, V-H4 and V-C8 cells. These findings argue against differences in drug uptake, metabolic activation or detoxification pathways being responsible for differential MMC toxicity in the three cell lines, since differences in any of these processes are expected to be reflected in different levels of DNA cross-linking in cell lines [20,21]. Thus, the enhanced sensitivity of V-H4 and V-C8 cells to MMC is rather due to cellular processes downstream of the initial DNA damage formation.

We found no evidence for significant repair of MMC cross-links at the level of the rRNA gene of either parental or mutant cell lines. Therefore, MMC-induced interstrand cross-links can subsist in hamster cells for extended periods of time. These data indicate that persistence of DNA interstrand cross-links is not responsible for the differential toxicity of MMC towards V-H4 and V-C8 cells. Moreover, we have shown that MMC-induced cross-links, originally formed in parental DNA, were also associated with newly replicated DNA. These findings are consistent with results from studies which also reported slow repair kinetics of MMC-induced cross-links [22] and distribution of cross-links in both parental and daughter DNA of human cells [23]. The presence of DNA interstrand cross-links in the replicated DNA after MMC treatment might be a consequence of a recombinational mechanism occurring during replication past cross-links, or a conversion of monofunctional MMC adducts to interstrand cross-links during the repair kinetics. Using an excision repair-deficient cell line, we have shown that, in addition to a first and rapid induction of MMC interstrand cross-links, there is a delayed two-step mechanism for MMC cross-link formation. The first alkylation step would yield monoadducts, and the second alkylation biadducts after some period of time [24]. These findings may explain discrepancies found in the literature concerning MMC interstrand cross-link repair studies in normal and FA cells [25,26], since cross-link detection would be the result of a combination of delayed formation and incision during the repair process. We therefore concluded that delayed formation associated with slow repair may explain the persistence of MMC-induced cross-links in V79, V-H4 and V-C8 hamster cell lines.

To be able to compare the repair characteristics of V-H4 and V-C8 cell mutants with those of the V79 parental cell line, we next examined the removal of cisplatin-induced cross-links from the rRNA gene. It was previously reported that cisplatin-induced interstrand cross-links are repaired equally efficiently in RNA polymerase II-transcribed genes, such as the DHFR gene, and RNA polymerase I-transcribed genes, such as the rRNA gene [27,28]. Our present results show that excision repair of cisplatin interstrand cross-links is efficient in resistant as well as in sensitive cell lines. This indicates that V-H4 and V-C8 hypersensitive mutants are not deficient in repair of interstrand cross-links, such as MMC-hypersensitive mutants of complementation group 1, which are defective in nucleotide excision repair and unable to excise DNA interstrand cross-links [5].

Finally, hypersensitive V-H4 and V-C8 cells could not be differentiated from resistant V79 cells by comparing their MMC- or cisplatin-induced cross-link repair efficiencies at the level of the rRNA gene. Therefore, we concluded that a defect in DNA excision repair of interstrand cross-links was not responsible for the cytotoxic effects of MMC and cisplatin in V-H4 and V-C8 hamster cells. Studies are under way in our laboratory to identify the cellular response(s) to cross-linking agents that is defective in these two hypersensitive mutants.

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