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Involvement of Apoptosis in Mitomycin C Hypersensitivity of Chinese Hamster Cell Mutants

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ABSTRACT. To elucidate the mechanisms of the mammalian cell defense against cross-linking agents, we studied previously cellular responses to mitomycin C (MMC) treatment in two MMC-hypersensitive hamster cell mutants' V-H4 and V-C8, as well as their parental cell line V79. In the present report, we investigated whether alterations in cell cycle checkpoints and induction of apoptosis could be responsible for the MMC hypersensitivity of the V-H4 and V-C8 mutant cell lines. First, we found that parental and mutant cells exhibited similar cell cycle responses to MMC concentrations of equivalent cytotoxicity, arguing against a defective cell cycle checkpoint in hypersensitive cell lines. In contrast, we showed that mutant cells underwent greater levels of apoptosis following MMC treatment than parental cells. These findings indicate that increased induction of apoptosis contributes to the hypersensitivity of V-H4 and V-C8 cells to the growth inhibitory effect of MMC. This differential apoptotic response was observed with both equimolar and equitoxic MMC doses and was specific to the cross-linking agent MMC, suggesting that control of the apoptotic process is altered in both MMC-hypersensitive mutants. The defective genes in V-H4 and V-C8 cells would then function in the regulation of an apoptotic pathway triggered by MMC-induced damage and independent of p53-mediated transcription. BIOCHEM PHARMACOL **59**;9:1101–1107, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. mitomycin C; mitomycin C hypersensitivity; DNA damage; apoptosis

Mitomycin C is a cancer chemotherapeutic agent which is used as single agent or in combination in the treatment of gastrointestinal adenocarcinomas, some head and neck cancers, and non-small cell lung cancers (for review, see [1]). MMC§ mainly alkylates the N-2 position of guanine, upon reductive activation cascade [2], to form either monoadducts or intra- or interstrand cross-links [3]. Since interstrand cross-links prevent DNA strand separation and can constitute complete blocks to DNA replication and transcription, the toxicity of MMC has been mainly correlated to the formation of these adducts [4]. However, although most of the chemical lesions produced by MMC *in vitro* are known, the molecular basis of its antitumor activity has yet to be understood. DNA repair efficiency and inhibition of DNA synthesis [5], as well as cell cycle checkpoint responses and induction of cell death by apoptosis [6], have been considered as the critical steps in toxicity induced by genotoxic bifunctional agents.

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§ *Abbreviations:* MMC, mitomycin C; TUNEL, TdT dUTP nick end-labeling; DAPI, 4′,6-diamidino-2-phenylindole; and FA, Fanconi's anemia.

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To elucidate the mechanisms of the mammalian cell defense against MMC and cross-linking agents in general, mutants specifically sensitive to MMC have been isolated in rodent cells [7]. The genetic and biochemical complexity of these processes is reflected by the existence of at least 8 complementation groups identified thus far among rodent cell mutants defective in this response [8, 9]. V-H4 and V-C8 cell mutants, representative of 2 different complementation groups, were isolated from V79 Chinese hamster cells [10, 11]. The V-H4 mutant cell line exhibits many characteristics of cells derived from FA patients [10, 12]. V-H4 cells show increased sensitivity towards cross-linking agents such as MMC (~30-fold more sensitive than the wild-type V79 cells) and cisplatin (~10-fold), but are not sensitive to UV light and X-rays [11]. The V-C8 mutant cell line also shows increased sensitivity towards the cross-linking agents MMC (~100-fold more sensitive than the wild-type V79 cells) and cisplatin (~40-fold), but is only slightly sensitive to UV (~2-fold) and X-rays (~2-fold) [11]. 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.

In this report, we investigated whether alterations in cell cycle checkpoints and/or induction of apoptosis could be responsible for the MMC hypersensitivity of V-H4 and

V-C8 mutant cell lines. Cell cycle analysis indicated that untreated V-H4 and V-C8 cell lines exhibited increased G2/M transit times and that treatment with MMC further increased the delay in the G2/M compartment. However, parental and mutant cell lines showed similar cell cycle distributions following equitoxic MMC doses, arguing against a defective cell cycle checkpoint in V-H4 and V-C8 cells. In contrast, we demonstrated that V-H4 and V-C8 cells underwent higher amounts of apoptosis following equimolar and equitoxic MMC treatments than their parental cell line V79, indicating that increased apoptosis contributes to MMC hypersensitivity in these 2 mutant cell lines.

MATERIALS AND METHODS

Cells and Culture Conditions

The MMC-sensitive mutants derived from Chinese hamster V79 cells, V-H4 and V-C8, have been described previously [10, 11]. V79, V-H4, and V-C8 cell lines were routinely grown in monolayer in Ham's F-10 medium (GIBCO) supplemented with 15% newborn bovine serum (GIBCO), penicillin (100 units/mL), and streptomycin (0.1 μg/mL). All incubations were at 37° in humidified 5% CO_2 atmosphere. Sensitivity of cells to MMC was assessed in a colony-forming growth assay. The MMC concentrations that produced 90% growth inhibition (IC_{90}) were 5 μg/mL for V79, 150 ng/mL for V-H4, and 55 ng/mL for V-C8 cells [10, 11].

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 (Aldrich) or vincristine (Aldrich) for 1 hr at the indicated concentrations at 37°. After drug exposure, cells were washed with PBS and then incubated in fresh medium. Cells were exposed to 254-nm UV irradiation in PBS for the appropriate length of time and then incubated in fresh medium. In each case, control and treated cells were handled in the same way, the only difference being omission of the drug treatment or the UV exposure.

Cell Cycle Analysis

Immediately after and then 16, 22, and 42 hr following drug treatment, 2×10^6 cells were washed once in PBS, trypsinized, and collected by centrifugation for 10 min at 1000 *g*. Cells were then resuspended in 600 μL lysis buffer (3.4 mM citrate trisodic, 0.1% Nonidet P40, 1.5 mM spermine tetrahydrochloride, 0.5 mM tris(hydroxymethyl)aminomethane), treated with Trypsin 30 μg/mL (10 min, room temperature), RnaseA 50 μg/mL (20 min, room temperature), and propidium iodide 0.25 mg/mL (10 min, room temperature). The stained nuclei were collected by FACScan (Becton Dickinson). After appropriate gating excluding debris, 10^4 events were analyzed using CellFit™ software (Becton Dickinson).

Detection of Apoptosis

The morphology of apoptotic cells was examined 48 hr after MMC treatment (500 ng/mL for 1 hr) by staining cells grown and fixed on coverslips with DAPI (Sigma) in PBS for 15 min at 37°. Fluorescence observation and image acquisition were performed with an intensified fluorescence microscope. For DNA fragmentation analysis, genomic DNA was extracted and purified from cells at 0, 24, and 48 hr after MMC treatment (500 ng/mL) for 1 hr. DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA) containing 10 μg/mL of RnaseA and incubated for 1 hr at 37°. Five micrograms of each sample was electrophoresed on 1.5% agarose gel.

Quantification of Apoptosis

Analysis of DNA fragmentation was performed using end-labeling of nicked DNA with biotin-conjugated dUTP by terminal transferase (TUNEL assay). Attached and floating cells were collected at 0, 24, 48, and 72 hr after drug or UV treatment, washed with PBS, and fixed in 0.35% formaldehyde in PBS (pH 7.4) for 15 min on ice. After washing in PBS, cells were resuspended in 70% ethanol and stored overnight at −20°. Cells (10^6 rehydrated cells per sample) were then incubated at 37° for 1 hr after being resuspended in 75 μL of TUNEL buffer (0.1 M sodium cacodylate [pH 7.0], 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 0.05 mg/mL of BSA, 10 units of terminal transferase [Boehringer Mannheim], and 0.5 nM biotin-16-dUTP). In control samples, terminal transferase or dUTP was omitted. Subsequently, cells were rinsed with PBS containing 0.1% Triton X-100 and resuspended in 100 μL of a staining buffer containing 2.5 g/mL of streptavidin–fluorescein (Boehringer Mannheim), 4× saline-sodium citrate buffer, 0.1% Triton X-100, and 5% (w/v) non-fat dry milk. Samples were incubated for another 30 min in the dark at room temperature, washed in PBS/0.1% Triton, and resuspended in 900 μL PBS containing 5 μg/mL of propidium iodide, 0.1% RNAse A. Following incubation for 15 to 30 min in the dark, stained cells were examined by flow cytometry. Data were acquired using a FACScan (Becton Dickinson) and analyzed with the LysisII™ software (Becton Dickinson). For each sample, 10^4 events were recorded.

RESULTS

Cell Cycle Distribution Following MMC Treatment

Cells that are hypersensitive to DNA cross-linking agents often exhibit greater delay in cell cycle progression than their more resistant counterparts. To investigate whether the V-H4 and/or V-C8 cells also exhibited this characteristic response, we analyzed the cell cycle progression in V79, V-H4, and V-C8 cells after MMC treatment. Cells

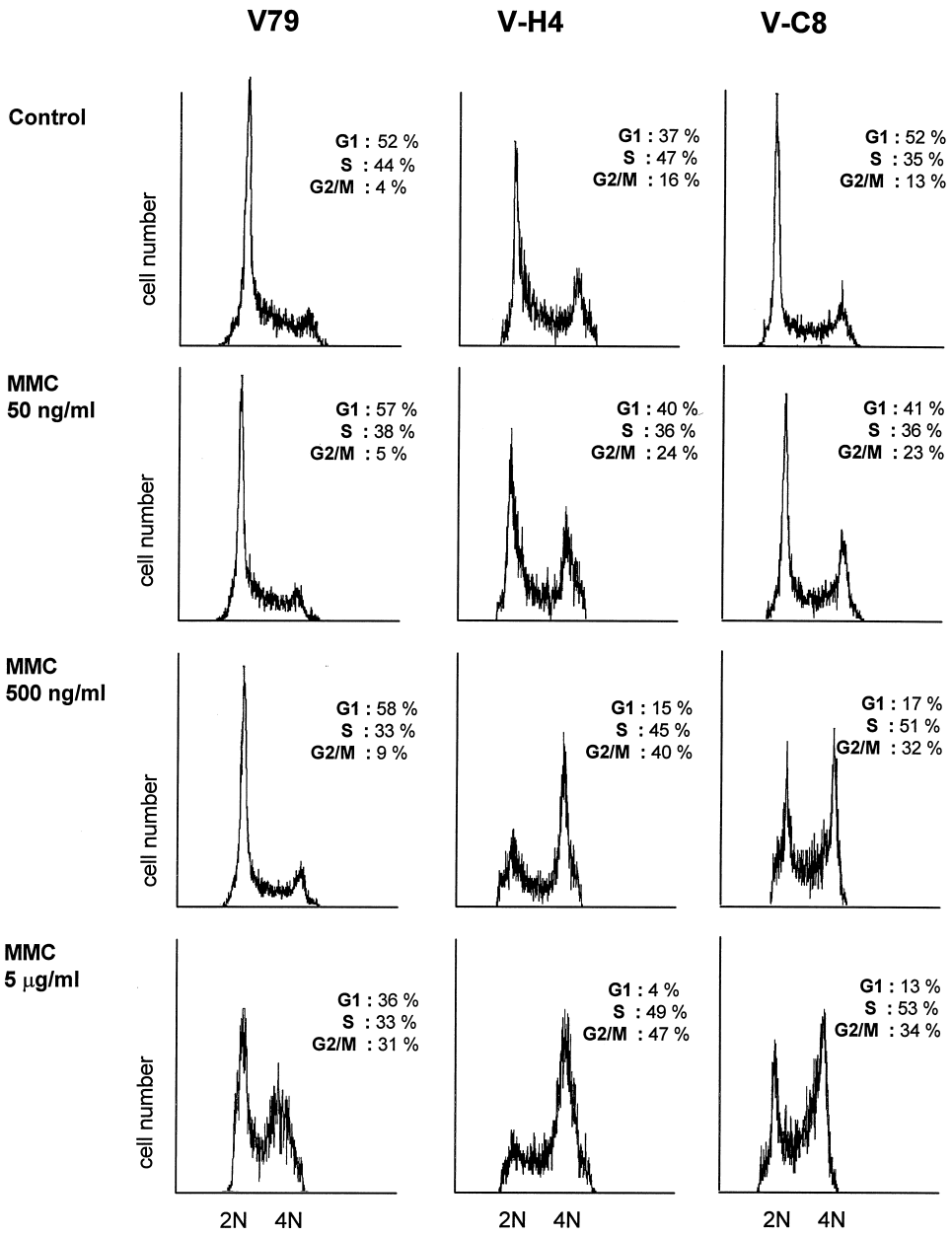

FIG. 1. Effect of MMC treatment on cell cycle distribution in hamster cells. V79, V-H4, and V-C8 cells were treated with indicated doses of MMC for 1 hr, stained with propidium iodide 22 hr after drug treatment, and collected by flow cytometry to determine the relative DNA content. Representative cell cycle distributions of untreated and treated cells are represented by histograms of DNA content, after appropriate gating excluding debris and sub-G1 peak of apoptotic cells. Data were acquired using a FACScan and analyzed with the CellFit™ software. 2N (G0/G1) and 4N (G2/M) DNA contents are shown.

were treated with increasing concentrations of MMC for 1 hr and then postincubated for various times in drug-free medium. They were finally stained with propidium iodide for analysis of DNA content by flow cytometry as described in Materials and Methods. Representative cell cycle distributions of parental V79 and mutant V-H4 and V-C8 cells postincubated for 22 hr after MMC treatment are shown in Fig. 1. Our results indicate that untreated V-H4 and V-C8 cells pass more slowly than do parental cells through the G2/M compartment of the cell cycle (Fig. 1). Incubating V-H4 and V-C8 cells with 50 or 500 ng/mL of MMC resulted in a marked accumulation of these mutant cells in G2/M phase (20 to 40%; Fig. 1). In contrast, these same concentrations of drug had no effect on the cell cycle progression of parental V79 cells (5 to 9% in G2/M phase; Fig. 1). When the dose of MMC was increased to 5 μg/mL, the 3 cell lines displayed a strong G2 arrest (30 to 50% of cells in G2/M phase; Fig. 1). These data show that 10- to 100-fold higher concentrations of MMC were required to arrest a similar fraction of parental V79 cells in G2/M phase compared to V-H4 and V-C8 cells. This order of magnitude is similar to that required for an equivalent MMC-induced

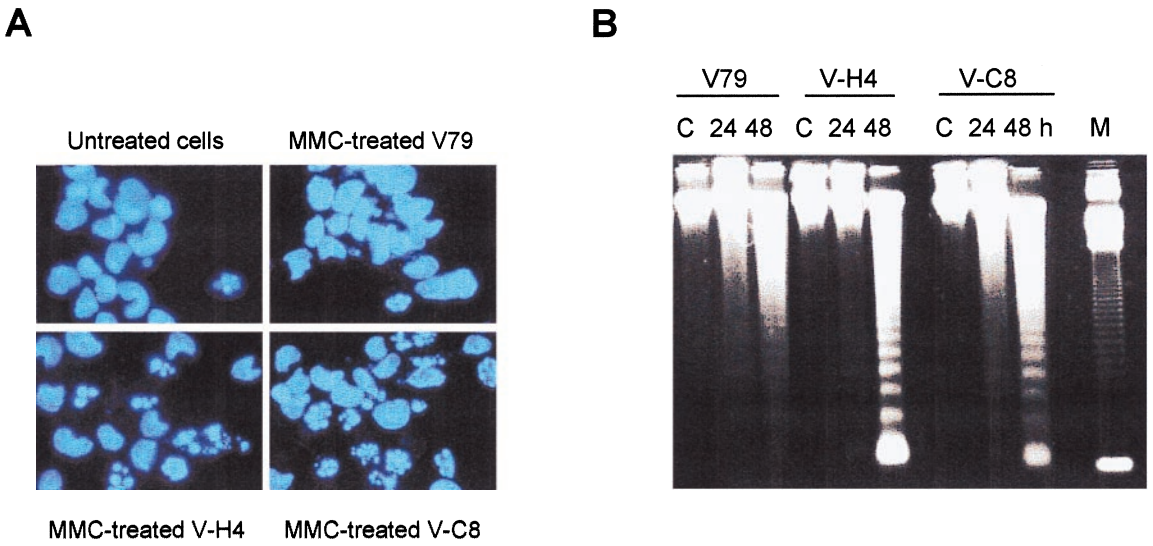

FIG. 2. Detection of MMC-induced apoptosis in hamster cells. (A) Cytological detection of apoptosis in V79, V-H4, and V-C8 cells. At 48-hr posttreatment with 500 ng/mL of MMC for 1 hr, cells were stained with DAPI and then examined by fluorescence microscopy. Photomicrographs show V-H4 and V-C8 cells undergoing apoptosis, with characteristic segregated and fragmented chromatin. (B) Time–course analysis of apoptotic DNA fragmentation in MMC-treated V79, V-H4, and V-C8 cells. Total genomic DNA was extracted at time points 0, 24, and 48 hr and subjected to electrophoresis on 1.5% agarose gel. M, 123-bp DNA ladder marker.

cytotoxicity in parental and mutant cell lines, since V-H4 cells are 30-fold and V-C8 cells 100-fold more sensitive than V79 cells. Finally, this analysis showed a greater susceptibility of V-H4 and V-C8 cells to MMC-induced cell cycle G2 arrest than their parental counterpart V79. However, parental and mutant cell lines exhibited similar cell cycle responses to MMC concentrations of equivalent cytotoxicity.

Detection of MMC-Induced Apoptosis

Mitomycin C, as well as other bifunctional chemotherapeutic agents, can cause cell death by apoptosis. We characterized MMC-induced cell death by apoptosis in these hamster cell lines by observation of the chromatin condensation, as revealed by fluorescence microscopy (Fig. 2A), and of the DNA fragmentation (Fig. 2B). Forty-eight hours after 500 ng/mL of MMC treatment for 1 hr, DAPI-stained hamster V-H4 and V-C8 cells displayed characteristic features of apoptosis, such as condensed chromatin and subnuclear bodies (Fig. 2A). Significantly more apoptotic cells were observed in V-H4 and V-C8 cultures than in the V79 population (Fig. 2A). To further show that V-H4 and V-C8 cells were undergoing apoptosis, gel electrophoresis of genomic DNA was performed. Forty-eight hours after MMC treatment, lysates from cells treated with 500 ng/mL of MMC for 1 hr were resolved by agarose gel electrophoresis and then stained with ethidium bromide. As shown in Fig. 2B, DNA cleavage with a characteristic pattern of internucleosomal ladder was observed in V-H4 and V-C8 cells. Although it is difficult to determine whether 10 or 25% of the total DNA in a particular lane is present in a nucleosomal ladder, mutant cell lines clearly showed a higher amount of apoptotic DNA fragmentation than did V79 cells (Fig. 2B). Taken together, these results indicate that hypersensitivity to MMC treatment in V-H4 and V-C8 cells is related to apoptosis.

Quantitation of MMC-Induced Apoptosis

As analysis of DNA fragmentation upon gel electrophoresis did not provide a suitable quantitative method of detection of apoptosis, a quantitation of the rate of MMC-induced apoptosis in parental and mutant cell lines was performed using the TUNEL assay. Apoptotic cells containing fragmented nuclear DNA were detected by *in situ* fluorescent dUTP-labeling of the 3′OH end of DNA by the terminal deoxynucleotidyl transferase, and quantified by flow cytometry as described in Materials and Methods. The amount of detected spontaneous apoptosis was low and similar in parental and mutant cell lines, i.e. only about 1 to 2% (data not shown). The rate of apoptosis in V79 and both mutant cell lines was quantitated after equitoxic MMC concentration exposure (80% survival after MMC treatment, i.e. 1 μg/mL of MMC for V79, 30 ng/mL of MMC for V-H4, and 10 ng/mL for V-C8 cells; Fig. 3A) or equimolar MMC concentration treatment (10 μg/mL of MMC for all cell lines; Fig. 3B). In both cases, the rate of apoptosis was significantly higher in V-C8 cells (50 to 60% at 72 hr; Fig. 3, A and B) than in either V-H4 cells (10 to 20% at 72 hr; Fig. 3, A and B) or parental V79 cells (only about 5%; Fig. 3, A and B). Although apoptosis is a very rapid and stochastic process that involves a small percentage of cells at any time, our results clearly showed a 10- to 12-fold increase (V-C8 cells) and a 2- to 4-fold increase (V-H4 cells) in the level of apoptotic cells after MMC treatment

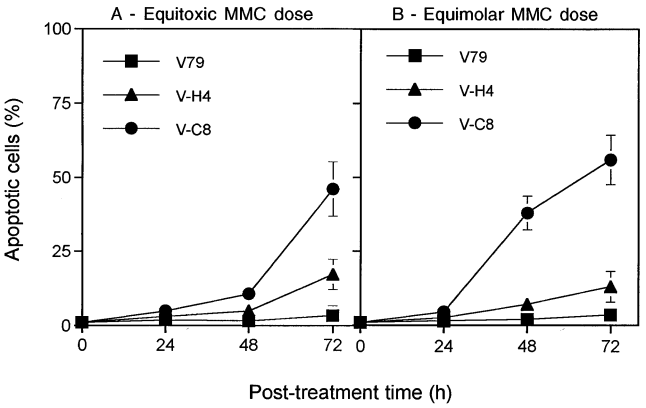

FIG. 3. Rate of MMC-induced apoptosis in hamster cells. The percent rates of MMC-induced apoptosis of V79, V-H4, and V-C8 cells treated for 1 hr with equitoxic concentrations of drug giving 80% cell survival (1 μg/mL of MMC for V79, 30 ng/mL of MMC for V-H4, and 10 ng/mL of MMC for V-C8 cells) (A) or with equimolar concentration of drug (10 μg/mL of MMC) (B) are shown as a function of time. Data are presented as mean values ± SE derived from 3 independent TUNEL assays and were acquired using a FACScan and analyzed with the LysisII™ software.

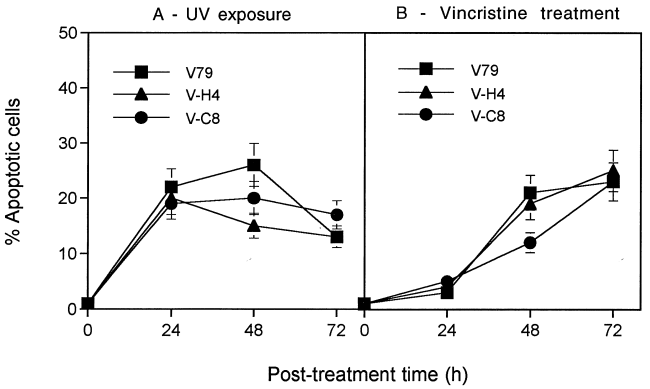

FIG. 4. Rate of UV- and vincristine-induced apoptosis in hamster cells. The percent rates of induced apoptosis of V79, V-H4, and V-C8 cells exposed to UV irradiation (15 J/m^2) (A) or treated with 100 nM vincristine for 1 hr (B) are shown as a function of time. Data are presented as mean values ± SE derived from 3 independent TUNEL assays and were acquired using a FACScan and analyzed with the LysisII™ software.

in sensitive cells. Similar differences between apoptosis rates in sensitive and resistant cells were found using lower equimolar MMC doses (500 ng/mL and 5 μg/mL; data not shown). Moreover, since use of the TUNEL assay allows us to visualize apoptotic cells as a function of their position in the cell cycle, we found that MMC-induced apoptosis occurred in all phases of the cell cycle (data not shown). Altogether, these data demonstrate that hypersensitivity of V-H4 and V-C8 cells to MMC is correlated with excessive induction of apoptosis. Furthermore, these two mutants are more prone to apoptosis than their parental counterpart, since they showed a greater amount of apoptosis induced by MMC concentrations of equivalent cytotoxicity.

An important step in the initiation of apoptosis is the accumulation of the tumor suppressor protein p53. Treatment of cells with a wide variety of DNA-damaging agents, including MMC, is known to induce an accumulation of p53. A recent study has indicated that the V79 p53 sequence contains two point mutations located within the presumed DNA-binding site [13]. We verified by Western blots that parental V79 cells and V-H4 and V-C8 mutants expressed high levels of basal p53 protein and did not accumulate further p53 protein after MMC treatment (data not shown). We may then conclude that the greater rate of MMC-induced apoptosis observed in V-H4 and V-C8 cells is independent of p53-mediated transcription.

MMC-Induced Specific Apoptosis

To examine the specificity of the apoptotic response for cross-linking agents such as MMC, we then tested the induction of apoptosis by a different genotoxic agent (UV light) and a non-genotoxic and antimitotic agent (vincristine), which targets tubulin. Using the TUNEL assay, we measured the rate of apoptosis in parental V79 and mutant V-H4 and V-C8 cell lines after UV irradiation (15 J/m^2) and vincristine treatment (100 nM for 1 hr). Both UV irradiation (Fig. 4A) and vincristine treatment (Fig. 4B) induced an increase in the fraction of apoptotic cells in the three cell lines, but there was no significant difference in apoptotic responses between parental and mutant cell lines (Fig. 4, A and B). Similar results were found using a higher UV fluence (30 J/m^2; data not shown). Thus, the deficient V-H4 and V-C8 gene products did not interfere with the apoptotic pathways induced by UV and vincristine. Therefore, the differential induction of apoptosis in V-H4 and V-C8 cells appears to be specific to MMC treatment. In addition, we have shown that V79 cells are not deficient in the induction of the apoptotic process itself, since both UV irradiation and vincristine induced apoptosis to a significant extent in these cells (Fig. 4, A and B). This may suggest that the signaling pathway evoked by MMC is different from that which is activated by UV irradiation in these hamster cells.

DISCUSSION

In the present report, we have studied cellular responses to mitomycin C in MMC-hypersensitive hamster V-H4 and V-C8 cells. There are many mechanisms by which hypersensitivity to bifunctional agents can be achieved in cells. The ability to induce and/or remove DNA interstrand cross-links may be an important factor contributing to MMC sensitivity of cells. In a previous report, we had compared 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 [14]. Using equimolar concentrations of drug, we measured equivalent amounts of MMC-induced DNA interstrand cross-links in the three cell lines [14]. These findings argued against differences in drug uptake, meta-

bolic activation, or detoxification pathways being responsible for differential MMC toxicity in the sensitive and resistant cell lines, since differences in any of these processes are expected to be reflected in different levels of DNA cross-linking in cell lines [15]. Furthermore, our previous data showed that persistence of DNA interstrand cross-links is not responsible for the differential toxicity of MMC towards V-H4 and V-C8 cells and that these mutants are not deficient in the initial step of excision repair of interstrand cross-links [14].

Hypersensitivity to genotoxic agents can also be achieved by dysregulation of the cell cycle checkpoints. Arrest of cells in G1 and G2 checkpoints in response to DNA damage is a well-described phenomenon in mammalian cells, presumably allowing DNA repair prior to DNA replication and mitosis, respectively (for review, see [16]). We have presented evidence herein that mutant V-H4 and V-C8 cells exhibit more prolonged cell cycle accumulation in G2 phase following equimolar MMC treatment than their parental counterpart. These data are consistent with several studies which have reported that DNA cross-link hypersensitive FA cells show prolonged accumulation in G2 after DNA damage [17] and that the FA polypeptide, FAC, binds to the cyclin-dependent kinase cdc2, which regulates the cell cycle progression from G2 to M phase in mammalian cells [18]. However, in response to equitoxic MMC concentrations, parental and hypersensitive mutant cell lines display equivalent accumulation in the G2/M compartment. Therefore, we concluded that the G2 arrest in V-H4 and V-C8 cells may reflect a secondary response to the excessive amount of damaged DNA following treatment with MMC, rather than a primary defect in the regulation of the G2/M transition. Such a hypothesis is in agreement with that of Heinrich *et al.* [19], who purported that the aberrant G2/M arrest in FAC lymphoblasts after MMC treatment represents a normal cellular response to the excessive DNA damage incurred in such cells and found no evidence of a cell cycle defect.

Induction of cell death by apoptosis can also enhance cytotoxicity to DNA-damaging agents. The data we present here show that mutant V-H4 and V-C8 cells undergo greater levels of apoptosis following MMC treatment than their parental counterpart V79. These results strongly suggest that increased induction of apoptosis contributes to the hypersensitivity of V-H4 and V-C8 cells to the growth inhibitory effect of MMC. Consistent with the phenotype of each cell line, a mutation in the *V-C8* gene has more drastic effects on MMC-induced apoptosis than inactivation of the V-H4 gene product. Based on our observations, V-H4 and V-C8 cells undergo MMC-induced apoptosis in all phases of the cell cycle, indicating that apoptosis is not a direct consequence of persistent G2/M phase accumulation. Interestingly, parental and mutant cell lines display differential amounts of apoptosis in response to equimolar as well as equitoxic MMC concentrations. The most likely explanation for our data is that both mutants are more prone to apoptosis than their parental counterpart. These findings, then, suggest that control of the apoptotic process is altered in both MMC-hypersensitive V-H4 and V-C8 cells.

Sustained p53 accumulation has been shown to initiate apoptosis in a variety of cell types [20]. However, a recent study has indicated that the parental V79 p53 sequence is mutated [13]. We have indeed observed that parental and mutant cell lines express high amounts of basal p53 protein and that MMC treatment does not mediate any further accumulation of this protein in these three cell lines. Thus, the V-H4 and V-C8 gene products would be involved in the regulation of an apoptotic pathway which is independent of p53-mediated transcription. These data are in agreement with the results of Strasser *et al.* [21], who showed apoptosis to be induced in MMC-treated lymphoid cells derived from p53 knockout mice and with studies from Kruyt *et al.* [22], who suggested that the FA group C gene product may function in an as-yet-unidentified p53-independent apoptosis pathway.

Interestingly, the induced cell death process that we report here is specific to the cross-linking agent MMC, since UV irradiation and vincristine treatment induced similar fractions of apoptotic cells in parental and mutant cell lines. These results are consistent with previous data showing that V-H4 and V-C8 cells are hypersensitive to MMC, but not (V-H4 cells) or only slightly (2-fold for V-C8 cells) sensitive to UV light [11]. Thus, our findings suggest that the defective genes in V-H4 and V-C8 cells function in the regulation of an apoptotic cascade triggered by MMC-induced damage. These gene products may be involved at different levels of the activation of the apoptotic pathway: (a) either in the specific detection of the initial DNA damage, being a "DNA interstrand cross-link sensor," in the detection of a repair intermediate such as a DNA strand break, or even in the detection of oxygen radical intermediates produced by cellular detoxification of MMC [23]; or (b) directly in the signal transduction cascade mediating apoptosis. Because of the specific MMC-induced apoptotic response, our data suggest that V-H4 and V-C8 gene products are rather involved in an upstream event that signals the initiation of apoptosis and regulates susceptibility to MMC, but not in the late proteolytic cascade of the process. Finally, evidence for phenotypic similarities between these MMC-hypersensitive hamster cell mutants and FA cells is further supported by the fact that high susceptibility to MMC-induced apoptosis is common for FA-C cells [22, 24], although opposite results were also obtained in studies using FA cells from different complementation groups in which no apoptosis was observed after exposure to MMC [25, 26]. Involvement of FA gene products in different pathways regulating apoptosis may explain these various findings.

In summary, MMC-hypersensitive V-H4 and V-C8 hamster cell lines provide a useful system to study the induction of MMC-induced apoptosis in mammalian cells. Furthermore, they provide a promising way to elucidate the defective molecular mechanism(s) involved in cells from

FA patients. Additional experiments will be needed to investigate the precise function(s) of the V-H4 and V-C8 gene products. Understanding of these mechanisms may be relevant to anticancer drug therapy, as apoptosis may be an important factor in drug responses.

References

1. Dorr RT and Von Hoff DD, Mitomycin C. In: *Cancer Chemotherapy Handbook*, 2nd Edn (Eds. Dorr RT and Von Hoff DD), pp. 717–726. Appleton & Lange, East Norwalk, 1994.
2. Tomasz M and Lipman R, Reductive metabolism and alkylation activity of mitomycin C induced by rat liver microsomes. *Biochemistry* **20:** 5056–5061, 1981.
3. Tomasz M, Mitomycin C: Small, fast and deadly (but very selective). *Chem Biol* **2:** 575–579, 1995.
4. Doll DC, Weiss RB and Issell BF, Mitomycin C: Ten years after approval for marketing. *J Clin Oncol* **3:** 276–286, 1985.
5. Plooy AC, van Dijk M, Berends F and Lohman PH, Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with *cis*-diamminedichloroplatinum(II). *Cancer Res* **45:** 4178–4184, 1985.
6. Demarcq C, Bunch RT, Creswell D and Eastman A, The role of cell cycle progression in cisplatin-induced apoptosis in Chinese hamster ovary cells. *Cell Growth Differ* **5:** 983–993, 1994.
7. Zdzienicka MZ and Simons JW, Mutagen-sensitive cell lines are obtained with a high frequency in V79 Chinese hamster cells. *Mutat Res* **194:** 239–249, 1987.
8. Jones NJ, Genetic analysis of mitomycin C-hypersensitive Chinese hamster cell mutants. *Mutagenesis* **9:** 477–482, 1994.
9. Telleman P, Overkamp WJ, van Wessel N, Studzian K, Wetselaar L, Natarajan AT and Zdzienicka MZ, A new complementation group of mitomycin C-hypersensitive Chinese hamster cell mutants that closely resembles the phenotype of Fanconi anemia cells. *Cancer Res* **55:** 3412–3416, 1995.
10. Zdzienicka MZ, Arwert F, Neuteboom I, Rooimans M and Simons JW, Chinese hamster V79 cell mutant V-H4 is phenotypically like Fanconi anemia cells. *Somat Cell Mol Genet* **6:** 575–581, 1990.
11. Overkamp WJ, Rooimans MA, Neuteboom I, Telleman P, Arwert F and Zdzienicka MZ, Genetic diversity of mitomycin C-hypersensitive Chinese hamster cell mutants: A new complementation group with chromosomal instability. *Somat Cell Mol Genet* **19:** 431–437, 1993.
12. Arwert F, Rooimans M, Westerfeld A, Simons JW and Zdzienicka MZ, The Chinese hamster cell mutant V-H4 is homologous to Fanconi anemia (complementation group A). *Cytogenet Cell Genet* **56:** 23–26, 1991.
13. Chaung W, Mi LJ and Boorstein RJ, The p53 status of Chinese hamster V79 cells frequently used for studies on DNA damage and DNA repair. *Nucleic Acids Res* **25:** 992–994, 1997.
14. Larminat F, Cambois G, Zdzienicka MZ and Defais M, Lack of correlation between repair of DNA interstrand cross-links and hypersensitivity of hamster cells towards mitomycin C and cisplatin. *FEBS Lett* **437:** 97–100, 1998.
15. Dulhanty AM, Li M and Whitmore GF, Isolation of Chinese hamster ovary cell mutants deficient in excision repair and mitomycin C bioactivation. *Cancer Res* **49:** 117–122, 1989.
16. Carr AM and Hoekstra MK, The cellular responses to DNA damage. *Trends Cell Biol* **5:** 32–40, 1995.
17. Dutrillaux B, Aurias A, Dutrillaux AM, Briot D and Prieur M, The cell cycle of lymphocytes in Fanconi anemia. *Hum Genet* **62:** 327–332, 1982.
18. Kupfer GM, Yamashita T, Naf D, Suliman A, Asano S and D'Andrea AD, The Fanconi anemia polypeptide, FAC, binds to the cyclin-dependent kinase, cdc2. *Blood* **90:** 1047–1054, 1997.
19. Heinrich MC, Hoatlin ME, Zigler AJ, Silvey KV, Bakke AC, Keeble WW, Zhi Y, Reifsteck CA, Grompe M, Brown MG, Magenis RE, Olson SB and Bagby GC, DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. *Blood* **91:** 275–287, 1998.
20. Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T, p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362:** 847–849, 1993.
21. Strasser A, Harris AW, Jacks T and Cory S, DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell* **79:** 329–339, 1994.
22. Kruyt FA, Dijkmans LM, van den Berg TK and Joenje H, Fanconi anemia genes act to suppress a cross-linker-inducible p53-independent apoptosis pathway in lymphoblastoid cell lines. *Blood* **87:** 938–948, 1996.
23. Doroshow JH, Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. *Proc Natl Acad Sci USA* **83:** 4514–4518, 1986.
24. Marathi UK, Howell SR, Ashmun RA and Brent TP, The Fanconi anemia complementation group C protein corrects DNA interstrand cross-link-specific apoptosis in HSC536N cells. *Blood* **88:** 2298–2305, 1996.
25. Monti D, Macchioni S, Guido M, Pagano G, Zatterale A, Calzone R, Cossarizza A, Straface E, Malorni W and Franceschi C, Resistance to apoptosis in Fanconi's anaemia. An *ex vivo* study in peripheral blood mononuclear cells. *FEBS Lett* **16:** 365–369, 1997.
26. Ridet A, Guillouf C, Duchaud E, Cundari E, Fiore M, Moustacchi E and Rosselli F, Deregulated apoptosis is a hallmark of the Fanconi anemia syndrome. *Cancer Res* **57:** 1722–1730, 1997.