Differential Activation of p53 by the Various Adducts of Mitomycin C*

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Mitomycin C (MC) is a cytotoxic chemotherapeutic agent that causes DNA damage in the form of DNA cross-links as well as a variety of DNA monoadducts and is known to induce p53. The various DNA adducts formed upon treatment of mouse mammary tumor cells with MC as well as 10-decarbamoyl MC (DMC) and 2,7-diaminomitosene (2,7-DAM), the major MC metabolite, have been elucidated. The cytotoxicity of DMC parallels closely that of MC in a number of rodent cell lines tested, whereas 2,7-DAM is relatively noncytotoxic. In this study, we investigate the ability of MC, DMC, and 2,7-DAM to activate p53 at equidose concentrations by treating tissue culture cell lines with the three mitomycins. Whereas MC and DMC induced p53 protein levels and increased the levels of p21 and Gadd45 mRNA, 2,7-DAM did not. Furthermore, MC and DMC, but not 2,7-DAM, were able to induce apoptosis efficiently in ML-1 cells. Therefore the 2,7-DAM monoadducts were unable to activate the p53 pathway. Interestingly, DMC was able to initiate apoptosis via a p53-independent pathway whereas MC was not. This is the first finding that adducts of a multiadduct type DNA-damaging agent are differentially recognized by DNA damage sensor pathways.

Mitomycin C (MC) (Chart 1), a natural antibiotic and cytotoxic cancer chemotherapeutic agent is used in the clinical treatment of several human malignancies (1). MC induces DNA damage in the form of DNA cross-links and monofunctional DNA alkylating products in various bacterial and mammalian cells and tissues (2–7). Treatment of mammalian cells with MC causes an increase in the cellular p53 level (8). In cells, MC is enzymatically reduced, yielding reactive species that are capable of producing radicals through redox cycling, as well as a variety of DNA adducts. Six major adducts are formed as shown in Figure 1. In addition, various metabolic molecular structures have been elucidated (4). Very recently, the MC derivative decarbamoyl mitomycin C (DMC; Chart 1) was shown to generate an array of DNA cross-links and monoadducts in EMT6 mouse mammary tumor cells that had similar or identical structures to those formed with MC.2 One of these, a monoadduct that is a stereoisomer of one of the monoadducts formed by MC is formed predominantly upon treatment with DMC.2 In contrast, another mitomycin derivative, 2,7-diaminomitosene (2,7-DAM), the major metabolite of MC in tumor cells (10), forms only monofunctional DNA adducts in the same cell line (4, 11). A comparison of the three drugs with respect to their DNA cross-linking and monoalkylating activities and their specific DNA adducts formed in the EMT6 cells is summarized in Table I and the corresponding DNA adducts are shown in Chart 2.

Comparative studies of the cytotoxicity of the same three mitomycins indicated that MC and DMC have closely similar cytotoxicities (12–14), whereas 2,7-DAM is essentially noncytotoxic in the cell lines tested (11, 15). Because the cytotoxicity of these drugs correlates with the frequencies of the DNA cross-link adducts but not with the DNA monoadducts, these findings point to cross-links as being the lesions primarily responsible for the cytotoxicities of the mitomycins.2 It is therefore apparent that the specific structures of the various DNA adducts play a differential role in the cytotoxicity of MC. The basis for a differential cytotoxic activity of the individual DNA adducts of MC is not known. However, p53 induction has been correlated in many instances with the cytotoxic activity of chemotherapeutic drugs (16). Although it was known that treatment of mammalian cells with MC results in an increase in the level of p53 (17) the outcome on p53 induction by DMC and 2,7-DAM had not been investigated. Little is known about how particular DNA adducts can activate a signal transduction program that culminates in p53 accumulation. Furthermore, studies focused on the cytotoxic activities of MC, DMC, or 2,7-DAM have not addressed the possible involvement of the tumor suppressor p53 in mediating the cytotoxic or apoptotic response. It may be hypothesized that the cytotoxic mechanism of MC is mediated, at least in part, by the tumor suppressor protein p53 (17, 18). The ability of DNA adducts to induce p53 has been implicated by the fact that agents that generate multiple type DNA adducts also cause the accumulation of p53.

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The abbreviations used are: MC, mitomycin C; DMC, decarbamoyl mitomycin C; 2,7-DAM, 2,7-diaminomitosene; RT, reverse transcriptase; PBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; pAb, polyclonal antibody; PARP, poly(ADP-ribose)polymerase; MTT, 3-(2,5-diphenyltetrazolium bromide).
However, it has been difficult to recognize a general correlation between cytotoxicities and the induction of p53 thus far (Ref. 21 and references therein). The lack of cytotoxicity of 2,7-DAM in contrast to MC and DMC may be explained by the hypothesis that 2,7-DAM-DNA monoadducts are unable to induce a signal transduction pathway that culminates in the induction of p53. We tested this hypothesis by comparing the induction of p53 in a wild-type p53 containing myeloid leukemia cell line (ML-1) by MC, DMC, and 2,7-DAM. We report here a dramatic differential p53 response in ML-1 cells treated with MC and DMC versus treatment with 2,7-DAM. We further show that a human myeloid leukemia cell line (K562), lacking functional p53 is resistant to MC but not DMC cytotoxic effects.

**MATERIALS AND METHODS**

**Reagents—**Camptothecin, propidium iodide, MTT assay reagents, and anti-actin were purchased from Sigma. Zeocin was purchased from Invitrogen. RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen. D. M. Vyas (Bristol-Myers Squibb Co.) supplied MC. 2,7-DAM and DMC were synthesized from MC as previously described (12, 22).

**Cell Culture—**The ML-1 cells were a generous gift from Michael Kastan. This myeloid leukemia cell line was grown in RPMI 1640 with 10% FBS and 5% CO2. The K562 leukemia cell line was purchased from ATCC and does not contain p53 (23). The cells were seeded at a density of 2.5 x 10^5/ml and exponentially growing cells were used in all experiments.

**MTT Cytotoxicity Assay—**ML-1 or K562 cells were grown in 1× RPMI supplemented with 10% FBS. Exponentially growing cells were seeded at 2.5 x 10^5/ml in 24-well plates and either left untreated or treated with graded dosages of MC, DMC, or 2,7-DAM for 24 h. Cells were spun down and re-suspended in 0.5 ml of MTT containing medium (0.5 mg/ml), and incubated at 37 °C for 1 h. Cells were spun down and re-suspended in 1 ml of 0.04 N HCl in isopropyl alcohol to lyse the cells. After 5 min at room temperature, samples were spun down and 250-μl aliquots were used for absorbance measurements. Cell viability was measured as the difference in the absorbance 550 and 620 nm. The assay detects living, but not dead cells. Data are expressed as a percent of the control healthy growing strain.

**Flow Cytometry—**FACS analysis was carried out on a BD Biosciences scan. Cells were spun down at 2300 rpm for 7 min, washed twice with phosphate-buffered saline, and resuspended in 20 ml of phosphate-buffered saline containing 2% bovine serum albumin and 0.1% NaN3. Ethanol (9 ml) was then added dropwise while vortexing. Propidium iodide staining and RNase treatment were carried out at 37 °C for 30 min 24 h prior to flow cytometry.

**Nuclear Extract Preparation—**Nuclear extract was prepared using a variation on the Dignam Protocol (24). Cells were spun down and resuspended in 5 packed cell pellet volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). They were then put on ice for 10 min prior to centrifugation for 10 min at 2,000 rpm. The pellet was resuspended in 2 packed cell pellet volumes of buffer A (volume prior to the initial wash). The cells were run through a 25-gauge needle twice and nuclei were then spun down at 2,000 rpm for 10 min followed by an additional 20-min spin at the 15,000 rpm. The pellet was resuspended in 10^6 cells per 3 ml of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) by running it through a 25-gauge needle twice. The suspension was rocked gently for 30 min at 4 °C. The extract was centrifuged for 30 min at 15,000 rpm and the supernatant aliquots were stored at −80 °C.

**TABLE I**

| Drug     | Monoadduct frequency | Cross-link frequency | Specific DNA adducts formed (Chart 2) |
|----------|----------------------|----------------------|---------------------------------------|
| MC       | 1.4 x 10^-5          | 4.8 x 10^-7          | +                                    |
| DMC      | 5.3 x 10^-5          | 3.4 x 10^-7          | +                                    |
| 2,7-DAM  | 9.5 x 10^-6          | 0                    | -                                    |

* From data in Ref. 9; error limits are omitted.

**CHART 1. Structure of mitomycins.**

**CHART 2. The major DNA adducts formed in MC-treated mouse mammary tumor cells.**
Specific Mitomycin C Monoadducts Do Not Activate p53

MC and DMC treatment but not treatment with 2,7-DAM results in the transcriptional activation of p53 target genes. Quantitative real-time RT-PCR with molecular beacons was used to analyze p21<sup>wt</sup> (A) and gadd45<sup>B</sup> (B) expression. Results were normalized using the control samples and the glyceraldehyde-3-phosphate dehydrogenase values to give relative units of mRNA induction. Bars represent the -fold induction of p21 and gadd45 as shown. Results are representative of two independent experiments.

**RESULTS**

**MC and DMC but Not 2,7-DAM Can Induce p53 Nuclear Accumulation**—The noncytotoxic effect of 2,7-DAM suggested that the DNA monoadducts produced in cells after treatment with this compound were unable to induce a signal transduction pathway that culminated in the induction of p53. We tested this hypothesis by treating the ML-1 cell line (a well-known model system for p53 studies) with MC and DMC, as well as with two other drugs shown previously to induce p53 (17). Treatment was carried out with the mitomycins at a concentration of 5 μM for 3 and 6 h, and nuclear extracts were prepared and analyzed by Western blot as shown in Fig. 1. Both MC and DMC induced a robust stabilization of p53 (Fig. 1, lanes 2–5) at both time points, whereas no p53 stabilization was detected in cells that had been treated with the same concentration of 2,7-DAM (Fig. 1, lanes 6 and 7). Higher doses of 2,7-DAM (50–100 μM) also failed to induce detectable levels of p53 (data not shown). It can be stated with high certainty that 2,7-DAM was unable to induce p53 as the Western blot analysis was carried out using a mixture of p53 monoclonal antibodies specific to the central domain as well as the carboxyl and amino terminal regions of p53.
terminus (pAb240, pAb421, and pAb1801, respectively). It is therefore highly unlikely that any p53 induced by 2,7-DAM would go undetected by the Western blot procedure employed. Both MC and DMC were able to induce p53 to levels comparable with those induced by treatment of the ML-1 cells with camptothecin (a topoisomerase targeting poison) and Zeocin (a member of the family of bleomycins) (Fig. 1, lanes 8–11).

**MC and DMC Treatment but Not Treatment with 2,7-DAM Results in the Transcriptional Activation of p53 Target Genes—**
The tumor suppressor p53 is a transcription factor that activates a multitude of downstream target genes (27). Two p53 downstream target genes that are well characterized are p21^{WAF1/CIP1} and gadd45 (28, 29). We analyzed the transactivation of the endogenous p21 and gadd45 genes in the ML-1 cell line in response to the chemotherapeutic drug treatments shown above. The drugs able to result in the stabilization of p53 (MC, DMC, Cpt, and Zeocin) also induced significant transactivation of the endogenous p21 and gadd45 genes as monitored by RT-PCR with molecular beacons (Fig. 2, A and B). The ML-1 cells treated with 2,7-DAM on the other hand showed very limited transactivation of the endogenous p53 target genes tested and this limited activation decreased at the longer time point (Fig. 2, A and B).

**The p53 Induced by MC and DMC Can Bind to DNA—** The treatment of ML-1 cells with both MC and DMC was able to induce p53 and also resulted in the activation of p21 and gadd45 transcription. This suggested that treatment of the ML-1 cells with both drugs resulted in stabilization of the p53 species that were able to bind to DNA. We analyzed the DNA binding ability of p53 in nuclear extracts derived from ML-1 cells treated with MC, DMC, and 2,7-DAM by EMSA (Fig. 3A). Although MC and DMC form different DNA adducts (Table I) both resulted in an increase in the level of p53 (Fig. 1) and an increase in the p53 DNA binding ability (Fig. 3A, lanes 2 and 3) when ML-1 cells were treated with a drug concentration of 5 μM for 6 h. Not surprisingly, no p53-specific gel shift was detected in nuclear extract derived from ML-1 cells treated with 5 μM 2,7-DAM for 6 h (Fig. 3A, lane 4). The pAb421 induced gel shift species detected in the ML-1 nuclear extract from drug-treated cells has been determined to be specific by competition analysis with nonlabeled specific and nonspecific oligonucleotide and a representative example is shown (Fig. 3B).

**MC and DMC but Not 2,7-DAM Are Able to Induce Apoptosis Efficiently in the ML-1 Cell Line Containing Wild-type p53—** Because treatment of ML-1 cells with MC and DMC, but not 2,7-DAM, was able to result in the stabilization of p53 we asked if this correlated with the ability of MC and DMC, but not 2,7-DAM, to induce apoptosis. ML-1 cells were treated with 5 μM MC, DMC, or 2,7-DAM for 24 h and the cells were then fixed and stained with propidium iodide. The samples were analyzed by FACS and compared with nondrug-treated cells to assess the increase in sub-G1 DNA content (Fig. 4A). Both the MC- and DMC-treated ML-1 cells showed a substantial increase in sub-G1 DNA content (although not as dramatic as that seen with Cpt), indicating that the ML-1 cells initiated apoptosis in response to these drugs. The ML-1 cells treated with 2,7-DAM on the other hand showed no increase in sub-G1 DNA content after 24 h of treatment, indicating that these cells were in fact not undergoing cell death. Apoptosis was also monitored by PARP cleavage and this further confirmed the induction of apoptosis by MC and DMC but not by 2,7-DAM treatment (Fig. 4D).

**DMC but Not MC or 2,7-DAM Is Able to Induce Apoptosis in the K562 Cell Line Lacking Functional p53—** To address the ability of MC and DMC to induce apoptosis in the absence of p53, the K562 cell line (a line without p53 (23)) was treated and analyzed in the same way. No substantial increase in sub-G1 DNA content was observed when the K562 cells were treated for 24 h with MC or 2,7-DAM (Fig. 4, B and E). Surprisingly, although K562 cells did not undergo apoptosis when treated with MC, a substantial increase in apoptotic cells resulted after DMC treatment as determined by FACS analysis and PARP cleavage (Fig. 4, B and E). This demonstrates that DMC was able to induce apoptosis in a p53-independent manner whereas MC was not. Therefore, although DMC is able to induce p53, its entire cytotoxic effect does not require p53.
Cytotoxicities of MC, DMC, and 2,7-DAM to the ML-1 (Wild-type p53) and K562 (p53 Null) Cell Lines—Cytotoxicity can be observed in the absence of apoptosis (30) and therefore the cytotoxicities of the panel of mitomycins were compared utilizing the ML-1 cells to confirm that 2,7-DAM was not cytotoxic. Cytotoxicity of the three mitomycins was monitored by the MTT cytotoxicity assay (Fig. 5) as well as by trypan blue exclusion (data not shown). Cytotoxicity was observed when ML-1 cells were treated with MC and DMC but not when they were treated with 2,7-DAM (Fig. 5A). Therefore 2,7-DAM treatment of ML-1 cells did not induce p53, did not induce apoptosis, and was not cytotoxic to the ML-1 cells. We also analyzed the cytotoxicity of these drugs in the p53 null cell line K562 to investigate their p53 independent cytotoxicities. Under these conditions 2,7-DAM was observed to be noncytotoxic, whereas MC demonstrated limited cytotoxicity and DMC was cytotoxic at the 5 and 10 μM drug concentrations (Fig. 5B). This correlated with the observation that DMC was able to induce PARP cleavage in the K562 cell (Fig. 4E). Although Fig. 5 demonstrates that DMC is not as cytotoxic to K562 cells as it is to ML-1 cells, it is clearly more cytotoxic to K562 cells than either MC or 2,7-DAM.

DISCUSSION

DNA Adducts Do Not Always Activate the p53 Pathway—It is often stated that DNA damage activates the tumor suppressor p53. Normally p53 is present at low levels and specific stimuli are able to elicit signal transduction pathways that allow for the stabilization of p53. Chemotherapeutic drugs that damage DNA function to a great extent by activating pathways that result in p53 stabilization (17). MC is a clinically relevant chemotherapeutic drug whose mode of action continues to be investigated. Here we demonstrate that, in contrast to the DNA adducts induced by MC and DMC, the monofunctional derivative of MC 2,7-DAM allows for DNA adducts that are unable to activate p53. Thus this form of DNA damage evades the p53 tumor suppressor pathway and is not cytotoxic. Previously it was shown that MC and DMC are equally cytotoxic in various rodent cell lines (12–14). The present work extends this finding to human myeloid leukemia cells. The cytotoxicity of MC has been attributed mainly to the generation of lethal DNA cross-links. DMC was initially thought to be devoid of DNA cross-linking activity, based on chemical considerations and on tests carried out in cell-free systems that employed chemical reductive drug activation (13, 31). Conse-
Specific Mitomycin C Monoadducts Do Not Activate p53

Fig. 5. MC, DMC, but not 2,7-DAM are cytotoxic in ML-1 cells, and only DMC is cytotoxic in K562 cells. MTT cytotoxicity assay of ML-1 (A) and K562 (B) cells. Exponentially growing ML-1 and K562 cells were either left untreated or treated with graded doses of MC, DMC, or 2,7-DAM for 24 h. Cells were then incubated in MTT-containing medium (0.5 mg/ml) at 37 °C for 1 h. Aliquots of cell lysate were used for absorbance measurements at 550 and 620 nm and the difference was calculated. Data are expressed as percent of control untreated cell line sample. Bars represent the S.E. ± mean of two independent experiments.

**A Relationship Exists between p53 Induction and Cytotoxicity of Mitomycins**—We show here that the ability of the two cytotoxic mitomycins to form DNA cross-links correlates with the ability of these drugs to induce the p53 pathway. The 2,7-DAM-DNA adducts do not contribute to cytotoxicity of MC (11, 15). In keeping with this lack of cytotoxicity of 2,7-DAM we show here that this compound is also unable to induce p53, suggesting that there is a connection between MC cytotoxicity and the ability to induce p53 in ML-1 cells. Indeed, of the three mitomycin drugs MC, DMC, and 2,7-DAM investigated here, only MC and DMC are significantly cytotoxic and only the same two drugs elicit the accumulation of p53. Therefore the ability of MC and DMC to signal for the induction of p53 may require the formation of DNA interstrand cross-links. However, the work presented here does not rule out the possibility that monoadducts, as long as they are not the ones produced by 2,7-DAM, might have the ability to activate the p53 pathway.

In fact, no direct relationship seems to exist between the structural nature of the DNA damage and stabilization of p53. UV light-induced DNA structural damage does not include DNA cross-links, yet p53 is stabilized as a result (17). O6-Methylguanine lesions of DNA, produced by the simple alkylating agent N-methyl-N′-nitro-nitrosoguanidine induce p53 efficiently (32); antibenzo[a]pyrene-diolepoxide, which forms bulky N2-guanine monoadducts also induces p53 (33). Thus, p53 induction can occur by specific types of monofunctional DNA lesions. It is likely to be mediated by the action of proteins specific to the particular structure of the lesion (see Ref. 32). Highly relevant to the present findings, it has been shown that DNA interstrand cross-links, formed by psoralen, induce "futile repair synthesis" (34), and the authors suggest that this process, rather than the cross-links per se, is responsible for inducing the p53 pathway. It is conceivable that the differential effects of the three mitomycin derivatives originate from the analogous phenomenon.

The fact that a mechanism exists in cells through reductive conversion of MC to 2,7-DAM to evade the cytotoxic potential of MC (11, 15) should be considered. It is likely that cells defective in this conversion will be more sensitive to MC treatment. Furthermore, derivatives of MC and/or MC analogues that cannot be metabolized (and thereby inactivated) to 2,7-DAM may be more effective in treating malignancies. Because of this DMC may be considered a more compelling clinical choice for use as a chemotherapeutic. DMC is cytotoxic, can induce p53 and does so in the absence of producing DNA adducts that can be evaded.

**Cytotoxicity and Induction of Apoptosis by DMC in the K562 Myeloid Leukemia Cell Line Lacking p53**—In contrast to MC, DMC was also capable of inducing apoptosis in the absence of p53 (as observed by drug treatment of the K562 myeloid leukemia cell line lacking p53). Although DMC was not as cytotoxic to K562 cells as it was to ML-1 cells, DMC was more cytotoxic to K562 cells than either MC or 2,7-DAM. This may be clinically relevant especially given the fact that over 50% of total human cancers have defective or mutated p53 genes (35). Perhaps this cytotoxicity results because DMC is able to activate an alternative DNA damage pathway that utilizes either p73 or BRCA1 (9, 36). This may be a reason for considering DMC for use in cancer chemotherapy. When choosing drugs to treat specific cancers it will be important in general to consider alternate DNA damage-mediated pathways.

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37. Specific Mitomycin C Monoadducts Do Not Activate p53 40519