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ROLES FOR OXIDATIVE STRESS AND POLY(ADP-RIBOSYL)ATION IN THE KILLING OF CULTURED HEPATOCYTES BY METHYL METHANESULFONATE

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Abstract—The mechanisms by which the methylating agent methyl methanesulfonate (MMS) kills cultured hepatocytes were studied. In an amino-acid-free Krebs–Ringer buffer (KRB), 1 mM MMS depleted the cells of glutathione (GSH) within 1–2 hr. Lipid peroxidation, as measured by the accumulation of malondialdehyde (MDA), followed, and over 70% of the cells died within 3–4 hr. The iron chelator deferoxamine and the antioxidant *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) prevented lipid peroxidation and death of the hepatocytes without any effect on the loss of GSH. 3-Aminobenzamide (ABA), a poly(ADP-ribose) polymerase inhibitor, also prevented the cell killing by attenuating the loss of GSH. In a culture medium containing amino acids and antioxidants (Williams' E medium, WEM), 1 mM MMS killed the hepatocytes more slowly, with 70% of the cells dying 8–12 hr after treatment. Lipid peroxidation accompanied the loss of viability. Deferoxamine and DPPD inhibited lipid peroxidation, while only partially protecting against the cell killing. ABA offered more protection and reduced the decline of GSH and decreased lipid peroxidation. ABA also reduced the extent of the depletion of both NAD and ATP that accompanied the cell killing by MMS in WEM. These data indicate that MMS killed the hepatocytes by different mechanisms depending on the culture conditions. In KRB, the toxicity of MMS was a consequence of oxidative cell injury that follows the depletion of GSH. In WEM, both oxidative injury and the action of poly(ADP-ribose) polymerase in response to DNA single-strand breaks contributed to the loss of viability.

Activation of poly(ADP-ribose) polymerase as a component of the repair of DNA damage has been implicated in the toxicity of a variety of environmental hazards, including alkylating agents [1–3], oxygen radicals [4, 5], X-irradiation [6], and UV-irradiation [7]. The nuclear enzyme poly(ADP-ribose) polymerase is activated by DNA strand breaks and catalyzes the transfer of an ADP-ribose moiety from NAD with the concomitant release of nicotinamide [8]. Repetition of this activity produces extended chains of ADP-ribose. Thus, extensive damage to DNA can lead to a substantial depletion of NAD. The nicotinamide released from NAD by poly(ADP-ribosyl)ation is reconverted to NAD in a reaction that consumes ATP [9]. In addition, NAD is an electron carrier in mitochondrial respiration. Thus, NAD depletion may also interrupt ATP production. It has been proposed that the metabolic consequences of the activation of poly(ADP-ribose) polymerase, namely the consumption of NAD and a consequent depletion of ATP, are critical determinants of the cell killing that occurs with many DNA-damaging agents [10].

Poly(ADP-ribosyl)ation was implicated in the killing of mouse L929 fibroblasts by three direct alkylating agents, *N*-methyl-*N*-nitrosourea (MNU)§, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and methyl methanesulfonate (MMS).‖ DNA single-strand breaks, NAD depletion, ATP depletion, and cell death occurred sequentially. An inhibitor of poly(ADP-ribose) polymerase, 3-aminobenzamide (ABA), prevented the depletion of NAD and ATP and the subsequent cell killing.‖

The same alkylating agents have been reported to kill isolated hepatocytes [11, 12]. Both MMS and MNNG rapidly deplete hepatocytes of glutathione (GSH), followed by evidence of lipid peroxidation and then cell death. Abrupt depletion of GSH sensitizes hepatocytes to their constitutive flux of partially reduced oxygen species [13, 14]. Such an oxidative stress is normally detoxified by GSH-dependent mechanisms. However, in the face of GSH depletion, these activated oxygen species are toxic. In this regard, with MNNG, but not MMS, the antioxidant promethazine prevented the killing of isolated hepatocytes [11]. With MMS, promethazine

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§ Abbreviations: ABA, 3-aminobenzamide; DMSO, dimethyl sulfoxide; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); GSH, glutathione; KRB, Krebs–Ringer buffer; LDH, lactate dehydrogenase; MDA, malondialdehyde; MMS, methyl methanesulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; and WEM, Williams' E medium.

‖ Mizumoto K and Farber JL, ATP depletion is the critical event in the cell killing by alkylating agents: Metabolic alterations that accompany poly(ADP-ribosyl)-ation. Manuscript submitted for publication.

prevents the accumulation of the products of lipid peroxidation, but does not prevent the cell killing [11].

Thus, the mechanism of the hepatotoxicity of MMS is not as clearly defined as that of MNNG. On the one hand, it is possible that with MMS a non-peroxidative mechanism of oxidative cell injury kills the hepatocytes. Alternatively, mechanisms of lethal cell injury unrelated to oxidative stress may be active, namely activation of poly(ADP-ribose) polymerase in response to methylation of DNA.

In the present paper, we have re-examined the mechanism of the killing of cultured rat hepatocytes by MMS. In particular, we compared the responses of the liver cells to this alkylating agent under two different culture conditions. Similar to the previous study [11], the hepatocytes were treated with MMS in a Krebs–Ringer buffer (KRB). In this situation, the hepatocytes were lethally injured by iron-dependent oxidative stress. By contrast, the cell killing by MMS in a culture medium containing amino acids and antioxidants (GSH, vitamin E, and ascorbic acid) was a consequence of both an oxidative stress and the activity of poly(ADP-ribose) polymerase.

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing approximately 200 g, were obtained from the Charles River Breeding Laboratory (Wilmington, MA). All animals were fed *ad lib.* and fasted overnight prior to use. Hepatocytes were prepared by collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) perfusion according to the method of Seglen [15]. Yields of $2–4 \times 10^8$ cells/liver with 90–95% viability by trypan blue exclusion were routinely obtained. The hepatocytes were plated in 25 cm^2 flasks (Corning Glass Works, Corning, NY) at a density of 1.33×10^6 cells/flask in 3 mL of Williams' E medium (WEM) (GIBCO Laboratories, Grand Island, NY) containing 9.1 IU/mL penicillin, 9.1 μg/mL streptomycin, 47 μg/mL gentamicin sulfate, 0.018 U/mL insulin, and 9% heat-inactivated (55° for 15 min) fetal bovine serum (JRH Biosciences, Kansas City, MO). After incubation for 2 hr at 37° in an atmosphere of 95% air–5% CO_2, the cultures were rinsed twice with a prewarmed *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, and 10.1 mM HEPES, pH 7.4) to remove unattached dead cells. Five milliliters of fresh WEM was added, and the cells were incubated overnight (16–20 hr). The cultures were then washed twice with 3 mL of prewarmed HEPES buffer and incubated in KRB (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 24 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose, pH 7.4) or in WEM minus fetal bovine serum with the additions indicated in the text. The number of living cells (trypan blue) was confirmed by counting in a hemocytometer before the experiment and ranged from 1.3 to 1.6×10^6 cells per flask.

Cell viability was determined by the release of lactate dehydrogenase (LDH) into the culture medium [16]. The percentage of dead cells was defined as the ratio of the LDH activity in the medium to the total LDH activity released upon the treatment of the cultures with Triton X-100. Cell viability measured in this manner correlated closely with that determined by the exclusion of trypan blue (data not shown).

MMS (Aldrich Chemical Co., Inc., Milwaukee, WI) was diluted in water and added to the cultures at a final concentration of 1 mM (0.5% vol). Deferoxamine (Ciba Pharmaceutical Co., Summit, NJ) was dissolved in deionized water and added to cultures at a concentration of 12 mM for 1 hr (4% vol). The cells were washed, and placed in 5 mL of either KRB or serum-free WEM containing 1 mM deferoxamine in order to chelate the iron mobilized from intracellular ferritin [17]. *N,N'*-Diphenyl-1,4-phenylenediamine (DPPD) (Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and added simultaneously with MMS at a final concentration of 1 μM (0.5% vol). ABA (Sigma) was dissolved in DMSO and added simultaneously with MMS at a final concentration of 10 mM (0.5% vol). DMSO alone had no effect on any of the manipulations shown in the text.

DNA single-strand breaks were measured by the fluorometric analysis of the rate of DNA unwinding as described previously [18]. The DNA was allowed to unwind in urea solution in 0.2 N NaOH for 12 min at 4°. An increase in single-strand breaks results in an increased rate of DNA unwinding and reflects a decrease in the content of double-stranded DNA. The data are expressed as the content of double-stranded DNA.

The content of NAD was determined by the methods described previously [19]. The ATP content of the hepatocytes was determined by the luciferin-luciferase (Sigma) method as described previously [20]. The GSH content of the hepatocytes was determined spectrophotometrically at 412 nm by the method of Sedlak and Lindsay [21]. Lipid peroxidation was determined by the accumulation of malondialdehyde (MDA) in the cultures by a modification of the method of Ohkawa *et al.* [22] without a butanol extraction.

Statistical analyses were performed by analysis of variance using Student–Newman–Keuls *post hoc* tests with the PC version of the SAS statistical package (version 6.04). Data are expressed as means ± SD.

RESULTS

Cell killing by MMS in Krebs–Ringer buffer. Hepatocytes cultured overnight in WEM were washed and placed either in fresh WEM without serum or in KRB. Over a course of 4 hr, the hepatocytes in WEM showed no loss of viability and essentially no evidence of lipid peroxidation (Table 1). By contrast, over the same time course, hepatocytes in KRB showed increased cell loss and evidence of lipid peroxidation (Table 1). Deferoxamine and DPPD prevented this accumulation of MDA and reduced the loss of cell viability, whereas the addition of ABA to the culture medium was without effect on viability or MDA formation (Table 1). These data imply that control cells in

Table 1. Cell killing and lipid peroxidation in hepatocytes treated with MMS in Krebs–Ringer buffer

Treatment	Dead cells (% of total)	MDA (nmol/10^6 cells)
Control (WEM)	3.8 ± 0.5	0.15 ± 0.03
Control (KRB)	16.0 ± 1.4	0.97 ± 0.13
Deferoxamine	5.2 ± 0.4	0.10 ± 0.01
DPPD	4.4 ± 0.4	0.06 ± 0.01
ABA	16.3 ± 0.4	0.95 ± 0.02
MMS	71.6 ± 1.0	3.45 ± 0.14
MMS + deferoxamine	23.7 ± 0.8	0.22 ± 0.02
MMS + DPPD	17.6 ± 1.0	0.05 ± 0.01
MMS + ABA	21.2 ± 4.3	1.05 ± 0.66

Where indicated, cultured hepatocytes were pretreated with 12 mM deferoxamine for 1 hr. After washing, cells were treated with 1 mM deferoxamine, 1 μM DPPD, or 10 mM ABA in the presence or absence of 1 mM MMS in KRB (except for the first line). The cellular content of MDA and the percentage of dead cells were determined after 4 hr. Data are the means ± SD of 3 flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

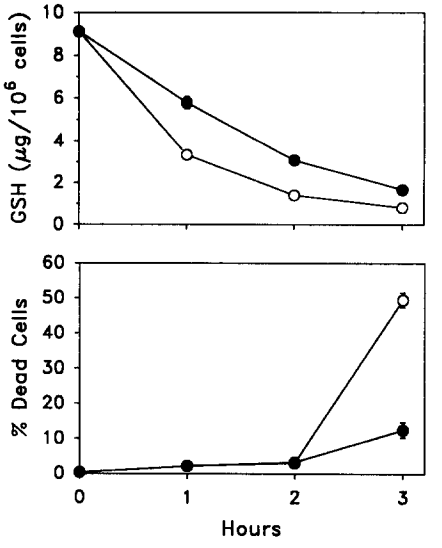

Fig. 1. Time–course of the depletion of GSH (upper panel) and the loss of viability (lower panel) of cultured hepatocytes treated with 1 mM MMS in the presence (●) or absence (○) of 10 mM ABA. The hepatocytes were treated in KRB. Results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

KRB suffer mild oxidative injury in the absence of any exogenous hazard.

In KRB, 1 mM MMS killed over 70% of the hepatocytes within 4 hr and caused an increase in lipid peroxidation (Table 1). Deferoxamine and DPPD reduced the cell killing and prevented the accumulation of MDA. ABA also reduced the loss of viability. However, less MDA accumulated with deferoxamine or DPPD than with ABA. With ABA the accumulation of MDA was the same as in control cells (in KRB).

Figure 1 details the time–course of the depletion of GSH and the killing of hepatocytes treated with 1 mM MMS in KRB. ABA was present or absent during the incubation. A decrease in GSH occurred within 1–2 hr and before cell killing. ABA reduced the rate and extent of decline of GSH, a result that correlated with the substantial reduction in the extent of cell killing (Fig. 1). By contrast, the protection afforded by deferoxamine and DPPD was not accompanied by any sparing of GSH depletion (data not shown).

The effects of the ABA concentration on the decrease in GSH and cell killing by MMS are detailed in Fig. 2. Increasing concentrations of ABA from 1 to 20 mM were associated with significantly increased contents of GSH 1 hr after treating the hepatocytes with 1 mM MMS ($P < 0.001$). The same concentrations of ABA produced increasing protection against the cell killing after 4 hr.

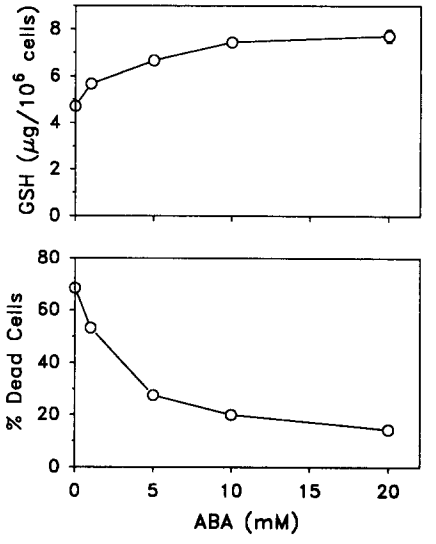

Fig. 2. Effect of increasing concentrations of ABA on the content of GSH (after 1 hr) and the loss of viability (after 4 hr) of cultured hepatocytes treated with 1 mM MMS in KRB. Results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

Role of DNA single-strand breaks in MMS toxicity in Krebs–Ringer buffer. The activation of repair mechanisms in response to the methylation of DNA by MMS is accompanied by the formation of single-strand breaks in DNA. DNA single-strand breaks were detectable (Table 2) prior to any loss of viability of the cells (Fig. 1). Table 2 also shows that the ability of deferoxamine, DPPD, or ABA to protect hepatocytes from the cell killing by MMS did not decrease the accumulation of DNA single-strand breaks. Repair of the methylation of DNA by MMS was not accompanied by a change in cellular content of NAD or ATP prior to the death of the cells (Fig. 3).

Table 2. Cell killing and DNA single-strand breaks in hepatocytes treated with MMS in Krebs–Ringer buffer

Treatment	% Double-stranded DNA
Control	62.8 ± 3.2
Deferoxamine	55.2 ± 8.4
DPPD	58.2 ± 3.5
ABA	62.0 ± 0.8
MMS	42.4 ± 7.0
MMS + deferoxamine	43.6 ± 1.9
MMS + DPPD	39.5 ± 1.5
MMS + ABA	32.9 ± 5.8

Where indicated, cultured hepatocytes were pretreated with 12 mM deferoxamine for 1 hr. After washing, cells were treated with 1 mM deferoxamine, 1 μM DPPD, or 10 mM ABA in the presence or absence of 1 mM MMS. The percentage of double-stranded DNA was determined after 2 hr. Data are the means ± SD of 3 flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

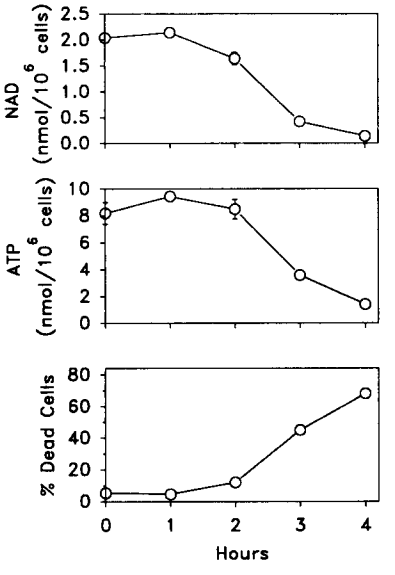

Fig. 3. Time–course of the effects of MMS on the content of NAD (upper panel), ATP (middle panel), and the loss of viability (lower panel) of cultured hepatocytes treated with 1 mM MMS in KRB. Results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

Cell killing by MMS in Williams' E medium. The toxicity of MMS differed in several respects when the hepatocytes were incubated in WEM rather than in KRB. Table 3 indicates that 1 mM MMS killed essentially the same number of cells in WEM as in KRB. However, the time–course of killing in WEM (Table 3) was much slower than in KRB (Table 1). As shown in Fig. 4 (lower panel), the cells first lost viability between 4 and 8 hr, and the number of dead cells continued to increase between 8 and 12 hr.

Table 3 also shows that the cell killing by MMS in WEM was accompanied by lipid peroxidation. In WEM, deferoxamine and DPPD reduced the cell killing by MMS by only 40%, despite the fact that both reduced the accumulation of MDA to the basal level. In WEM, ABA protected the hepatocytes to a greater extent than either deferoxamine or DPPD, a result that occurred despite an increase in lipid peroxidation ($P < 0.001$).

In WEM, MMS decreased cellular GSH concentrations by 76% after 2 hr. Deferoxamine and DPPD did not prevent this loss of GSH. By contrast, the reduced cell killing with ABA was accompanied by a decrease of only 59% ($P < 0.001$ from minus ABA) of GSH (Table 3), a result similar to the effect of ABA in KRB.

Figure 5 compares the time–course of changes in GSH in hepatocytes maintained in WEM or KRB. In WEM, control cells had increased cellular concentrations of GSH, whereas those in KRB showed no change. With 1 mM MMS, cells incubated in WEM had higher GSH concentrations than in KRB ($P < 0.001$ at 1 and 2 hr).

Role of DNA single-strand breaks in MMS toxicity in Williams' E medium. Figure 6 details the time–course of the accumulation of DNA single-strand breaks in hepatocytes treated with 1 mM MMS in WEM. Single-strand breaks accumulated during the first 4 hr and before any loss of viability (Fig. 4, lower panel). Table 4 shows that neither deferoxamine, DPPD, nor ABA prevented the accumulation of DNA single-strand breaks.

Hepatocytes treated with 1 mM MMS in WEM showed substantial depletion of NAD (Fig. 4, upper panel) prior to the loss of viability (lower panel). A significant decline in ATP (middle panel) and an increase in cell killing occurred by 8 hr. ABA reduced the loss of NAD (upper panel) at the same time that it protected against the cell killing by MMS (lower panel). There was also significantly more ATP ($P < 0.001$ at 8 hr) in the cells treated with ABA (middle panel). The protection by deferoxamine was without effect on the depletion of NAD and ATP (data not shown).

DISCUSSION

The data presented above define two independent mechanisms, oxidative stress and poly(ADP-ribosyl)ation, by which the alkylating agent MMS kills hepatocytes (Fig. 7). The particular pathway to lethal cell injury varies with the medium in which the hepatocytes are cultured.

Treatment with MMS in KRB kills the hepatocytes by an oxidative mechanism that depends on a cellular source of ferric iron and is accompanied by lipid peroxidation (Table 1). The iron chelator deferoxamine and the antioxidant DPPD effectively prevent the cell killing by MMS in KRB. An 85% decrease of GSH preceded the loss of viability (Fig. 1).

MMS is likely acting in KRB in a manner similar to our previous explanation of the killing of cultured hepatocytes by dinitrofluorobenzene [13], diethyl maleate [13], and allyl alcohol [14], all of which abruptly deplete GSH. The loss of GSH sensitizes

Table 3. Cell killing, lipid peroxidation, and GSH metabolism in hepatocytes treated with MMS in Williams' E medium

Treatment	% Dead cells	MDA (nmol/10^6 cells)	GSH (μg/10^6 cells)
Control	3.1 ± 0.8	0.16 ± 0.02	15.84 ± 0.32
Deferoxamine	2.7 ± 1.0	0.14 ± 0.01	ND
DPPD	3.4 ± 1.0	0.13 ± 0.01	ND
ABA	3.8 ± 1.0	0.17 ± 0.01	ND
MMS	71.5 ± 1.3	2.58 ± 0.19	3.74 ± 0.28
MMS + deferoxamine	43.1 ± 3.7	0.18 ± 0.10	3.48 ± 0.14
MMS + DPPD	42.8 ± 3.2	0.13 ± 0.01	3.29 ± 0.05
MMS + ABA	19.8 ± 3.2	0.88 ± 0.10	6.49 ± 0.19

Where indicated, cultured hepatocytes were pretreated with 12 mM deferoxamine for 1 hr. After washing, cells were treated with 1 mM deferoxamine, 1 μM DPPD, or 10 mM ABA in the presence or absence of 1 mM MMS. The cellular content of MDA and the percentage of dead cells were determined after 12 hr. GSH was determined after 2 hr. Data are the means ± SD of 3 flasks. Each experiment was repeated 2–3 times, and a representative one is shown. ND = not determined.

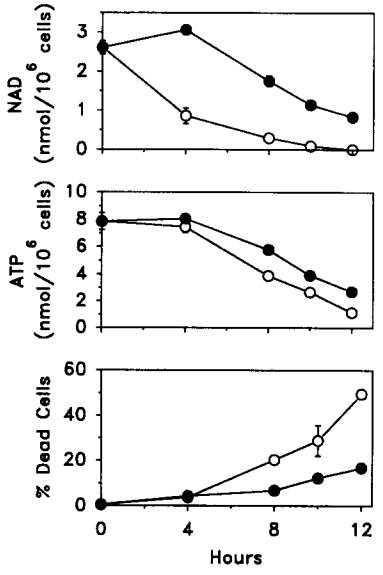

Fig. 4. Effect of ABA on the time–courses of the content of NAD (upper panel), ATP (middle panel), and the loss of viability (lower panel) of cultured hepatocytes treated with 1 mM MMS in WEM. Cells were treated in the presence (●) or absence (○) of 10 mM ABA. Results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

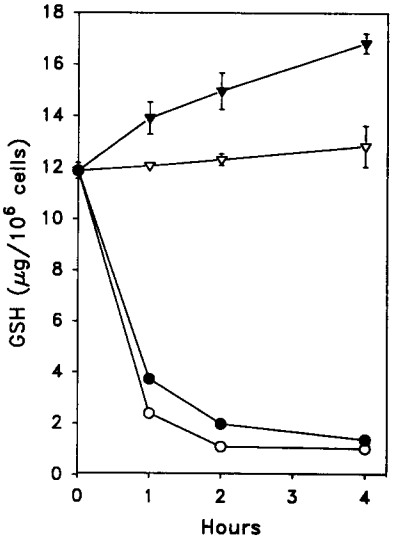

Fig. 5. Time–course of the content of GSH in cultured hepatocytes treated with 1 mM MMS in KRB (○) or in WEM (●). The GSH concentration in untreated control cells in KRB is shown by the open triangles (▽) or in WEM by the closed triangles (▼). The results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

hepatocytes to their constitutive flux of partially reduced oxygen species. Such an oxidative stress is normally detoxified by GSH-dependent mechanisms. However, in the face of GSH depletion, these activated oxygen species are toxic as a result of the iron-dependent formation of a potent oxidizing species (e.g. the hydroxyl radical) that acts as an inducer of lipid peroxidation.

Treatment of hepatocytes with MMS in KRB also resulted in the accumulation of single-strand breaks in DNA prior to the death of the cells (Table 2), presumably the result of repair processes in response to the methylation of bases. Oxidative stress did not cause these breaks, as deferoxamine did not prevent their appearance (Table 2). This repair reaction played no role in the cell killing, since NAD and ATP were not depleted prior to death of the cells (Fig. 3). Nevertheless, ABA effectively prevented the cell killing by MMS in KRB (Table 1). However, protection by ABA cannot be attributed to its action as an inhibitor of poly(ADP-ribose) polymerase.

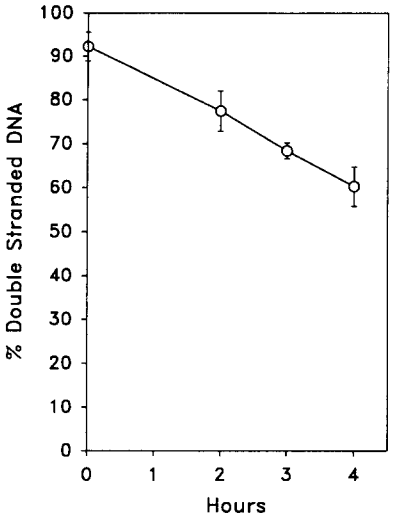

Fig. 6. Time–course of the accumulation of DNA single-strand breaks in the cultured hepatocytes treated with 1 mM MMS in WEM. Results are the means ± SD of 3 separate flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

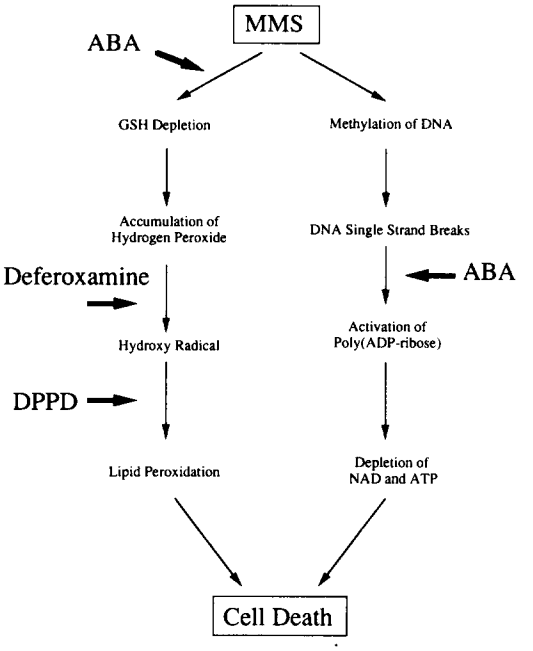

Fig. 7. Two mechanisms of the killing of cultured hepatocytes by MMS.

Table 4. DNA single-strand breaks in hepatocytes treated with MMS in Williams' E medium

Treatment	% Double-stranded DNA
Control	83.3 ± 1.3
Deferoxamine	81.9 ± 4.5
DPPD	83.3 ± 2.3
ABA	82.7 ± 3.3
MMS	49.1 ± 9.2
MMS + deferoxamine	37.1 ± 7.6
MMS + DPPD	31.8 ± 9.3
MMS + ABA	35.0 ± 9.7

Where indicated, cultured hepatocytes were pretreated with 12 mM deferoxamine for 1 hr. After washing, cells were treated with 1 mM deferoxamine, 1 μM DPPD, or 10 mM ABA in the presence or absence of 1 mM MMS. The percentage of double-stranded DNA was determined after 4 hr. Data are the means ± SD of 3 flasks. Each experiment was repeated 2–3 times, and a representative one is shown.

Rather, ABA acts to attenuate the decline of GSH by a mechanism that remains to be defined but may involve its action as a competitive nucleophile (Figs. 1 and 2). Thus, protection by inhibitors of poly(ADP-ribose) polymerase against oxidative cell killing does not necessarily imply the participation of this enzyme in the mechanisms of lethal injury.

*Mizumoto K and Farber JL, ATP depletion is the critical event in the cell killing by alkylating agents: Metabolic alterations that accompany poly(ADP-ribosyl)-ation. Manuscript submitted for publication.

† Mizumoto K and Farber JL, The inhibition of poly(ADP-ribose) polymerase has opposing effects on the interruption of cell growth and the loss of cell viability produced by *N*-methyl-*N*-nitrosourea. Manuscript submitted for publication.

MMS killed hepatocytes cultured in WEM more slowly than in KRB (Figs. 4 and 1, respectively), most likely the consequence of the presence of amino acids and antioxidants in WEM. Whereas MMS in KRB killed 71% of the hepatocytes within 3–4 hr, MMS in WEM required 12 hr to kill a comparable number of the cells. Furthermore, oxidative injury accounted for less than 50% of the cell killing in WEM (Table 3), as deferoxamine and DPPD reduced the number of dead cells from 71 to 43% of the total. Nevertheless, both agents completely prevented the accumulation of MDA. Prevention of lipid peroxidation was not accompanied by complete prevention of cell killing, indicating another mechanism of lethal injury.

The activity of poly(ADP-ribose) polymerase accounts for the larger percentage of hepatocytes killed by MMS in WEM. NAD depletion occurred within 4 hr and was followed by ATP depletion and cell death (Fig. 4). ABA reduced the decline of both NAD and ATP in parallel with the prevention of cell killing (Fig. 4).

Interestingly, ABA also decreased cell killing in WEM that is attributable to oxidative injury (Table 3), an action that was accompanied by a reduction in lipid peroxidation. Protection by ABA in WEM, as in KRB, is likely the consequence of an increased cellular content of GSH (Table 3). Thus, by virtue of its effect on both GSH metabolism and poly(ADP-ribose) polymerase, ABA reduced the cell killing by MMS in WEM from over 70% to less than 20% of the hepatocytes.

With the present study and three previous ones [23, *, †], we have detailed the role of poly(ADP-ribosyl)ation in toxic cell injury. In particular, we

have studied two differing hazards, oxidative stress [23] and alkylating agents*,†, in two different cell systems, primary cultures of rat hepatocytes and the L929 continuous line of mouse fibroblasts.

Oxidative stress kills cells by three different mechanisms: lipid peroxidation [24], non-peroxidative mitochondrial damage [25], and poly(ADP-ribosyl)ation [4, 5, 23, 26]. The particular mechanism that predominates depends on the cell type.

Hepatocytes are killed by oxidative stress as a result of the propensity of their membranes to undergo lipid peroxidation [24]. Alternatively, oxidative stress kills hepatocytes as a consequence of mitochondrial damage [25]. The dependence of hepatocytes on aerobic respiration probably renders them susceptible to oxidative mitochondrial damage.

There is no evidence that poly(ADP-ribosyl)ation plays a role in oxidative injury in hepatocytes. This conclusion holds when the hepatocytes are lethally injured by the exogenous oxidants *tert*-butyl hydroperoxide or hydrogen peroxide [19, 23]. Alternatively, when the oxidative stress resulted, as in the present report, from an inability of the hepatocytes to detoxify an endogenous flux of activated oxygen species, there was still no evidence that poly(ADP-ribosyl)ation participated in the mechanism of lethal cell injury (Fig. 3).

By contrast, other cell types are killed by oxidative stress as a result of poly(ADP-ribosyl)ation. The killing of cultured fibroblasts requires the participation of poly(ADP-ribose) polymerase [23]. Similarly, evidence exists for a role for poly(ADP-ribosyl)ation in the killing of lymphocytes [4], macrophages [4, 26], and endothelial cells [5] by oxidative stress.

With regard to the toxicity of MMS, the present report documents two mechanisms whereby this alkylating agent kills hepatocytes: oxidative stress with lipid peroxidation and DNA damage with poly(ADP-ribosyl)ation. The oxidative injury is likely a consequence of the depletion of GSH and the resulting inability to detoxify endogenous activated oxygen species. The contribution to lethal injury made by oxidative stress and poly(ADP-ribosyl)ation also varies with the cell type.

Whereas both mechanisms are active in hepatocytes, the treatment of fibroblasts with alkylating agents results in cell killing by a mechanism that depends exclusively on poly(ADP-ribosyl)ation.* The absence of oxidative cell injury with alkylating agents in fibroblasts may relate to their reduced dependence on aerobic respiration, a relative resistance of their membranes to lipid peroxidation, and differences in GSH metabolism.

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* Mizumoto K and Farber JL, ATP depletion is the critical event in the cell killing by alkylating agents: Metabolic alterations that accompany poly(ADP-ribosyl)-ation. Manuscript submitted for publication.

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