Cell Line-Dependent Changes in Sensitivity to Mitomycin C by 1-Methyl-3-isobutylxanthine Is Due to an Altered Intracellular Dose of the Anticancer Drug and/or to Changes in DT-diaphorase Activity

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Sensitization by 1-methyl-3-isobutylxanthine (MIX), a potent inhibitor of cAMP phosphodiesterase, of cellular sensitivity to mitomycin C (MC) was tested using various cell lines. Sensitization was seen with CHO cells and Balb/c 3T3 cells, but a decrease in sensitivity was seen with HeLa, K-balb and other cell lines. To measure the extent of crosslinks produced by MC, we used an alkaline elution assay. The extent of crosslinks was increased by MIX in CHO cells and decreased in K-balb cells. These changes in extent are well reflected by changes in sensitivity to MC in both cell lines. In CHO cells, an intracellular dose of MC was increased by MIX with no change in the rate of uptake or efflux of MC. There was a slight decrease in the dose in HeLa or K-balb cells. Among the enzymes which engage in the reductive activation of MC, we tested DT-diaphorase and NADPH-cytochrome P450 reductase using CHO, HeLa and K-balb cells. MIX had almost no effect on the activity of NADPH-cytochrome P450 in each cell line, whereas it suppressed the activity of DT-diaphorase, significantly in HeLa and K-balb cells, and slightly in CHO cells. All these facts indicate that MIX caused an increase in the extent of crosslinks *via* an increase in the intracellular dose of MC in CHO cells, and that these increases may lead to the sensitization. On the other hand, the decrease in the sensitivity shown in HeLa and K-balb cells may be due to the suppression of DT-diaphorase activity and/or to a decrease in MC dose. The effect of forskolin, another cAMP raising agent, on the sensitivity to MC was examined in CHO cells to test whether the sensitization by MIX involves the intracellular cAMP level. The treatment with forskolin had little effect, indicating that there was no such involvement.

Keywords mitomycin C; cellular sensitivity; 1-methyl-3-isobutylxanthine; crosslink; intracellular MC dose; cAMP level

Mitomycin C (MC) is an anticancer drug widely used as a treatment for cancer. The formation of crosslinks, especially DNA interstrand crosslinks, is considered a major lesion responsible for the MC cell killing effect.^{1,2)} Recently, it has been revealed, by exposing DNA to MC, that two neighboring guanine moieties in the complementary strands are involved in this crosslink formation.³⁾ DNA monoadducts and cytotoxic oxygen species are also produced and may have some role in the cell killing effect.^{4–10)} MC requires bioreductive activation to be cytotoxic. Several enzymes engage in this activation. However, how much these enzymes contribute to the activation varies depending on cell lines, extracellular pH, and pO₂.^{11–16)} Membrane permeability is also an important factor in determining cellular sensitivity to MC. Heat treatment during or just before MC exposure increases MC permeability and enhances cellular sensitivity to MC.¹⁷⁾

In an earlier study, we showed that treatment of CHO cells with 1-methyl-3-isobutylxanthine (MIX), an agent which raises cAMP levels, during MC exposure caused an enhancement of the cytotoxic action of MC.¹⁸⁾ The possibility of a repair system participating in the increased sensitivity appears to be very small, since MIX treatment after MC exposure had only a slight effect.¹⁸⁾ Two possible mechanisms, therefore, have been proposed to be involved in the enhancement; modification of enzyme activities concerning the bioactivation of MC, and changes in the intracellular dose of nonbound MC. We tested these possibilities and determined the extent of crosslinks as a major damage induced by MC. As MIX is well known to increase cAMP levels in cells, we used forskolin, another

cAMP increasing agent,¹⁹⁾ to test the possible participation of cAMP levels in the sensitization. To extend our observations to other kinds of cells, we performed some of the experiments done for CHO cells.

MATERIALS AND METHODS

Chemicals Mitomycin C (MC) was purchased from Kyowa Hakko Kogyo Co., Tokyo. 1-Methyl-3-isobutylxanthine (MIX), cytochrome c, tetrapropylammonium hydroxide, NADPH, FAD, dichloroindophenol, bovine serum albumin (BSA) and forskolin were obtained from Sigma Chem., St. Louis, U.S.A. Sodium *N*-lauroylsarcosinate (Sarkosyl) and Tween 20 were from Wako, Osaka and Nacalai Tesque, Kyoto, respectively. Eagle's minimum essential medium (MEM) was from Nissui, Tokyo, and calf serum (CS) and fetal bovine serum (FBS) were from Flow Lab., Australia. MC, MIX and forskolin were dissolved in dimethylsulfoxide (DMSO) and added to a culture medium at a final concentration of DMSO not exceeding 1%. For a culture without these chemicals, the same concentration of DMSO was added to the culture medium.

Cell and Cell Culture CHO, HeLa and V79 cells were maintained in our laboratory. Balb/c 3T3 was provided by Dr. A. Hakura, Osaka Univ., and K-balb established by Aaronson and Weaver²⁰⁾ was obtained from Dr. O. Niwa, Hiroshima Univ. Skin fibroblasts from human embryo (SH-21S) and a Fanconi's anemia patient (FAOnSa(5)) were established in this laboratory²¹⁾ and by Dr. M. S. Sasaki, Kyoto Univ., respectively. Human fibroblasts (SH-21S, FAOnSa(5)) were cultured in MEM

supplemented with 15% FBS in 5% CO₂ in air at 37 °C. Other cell lines were cultured in MEM with 10% CS.

Colony Formation Exponentially growing cells were exposed to MC at various doses for 60 min in the absence or presence of 1 mM MIX. After exposure to MC, the cells were washed twice with phosphate buffered saline (PBS), collected with trypsin, and replated for colony formation. The colonies were fixed with ethanol after culture for 7–14 d, depending on cell lines, and were counted after being stained with 3% Giemsa's solution.

Alkaline Elution Analysis The alkaline elution analysis was performed, as previously reported.²²⁾ Exponentially growing CHO or K-balb cells were incubated with 7.4 kBq/ml of [³H]thymidine (7.4 GBq/mmol, ICN Radiochem. Inc., CA) for 24 h to label the DNA, followed by 18 h of incubation in medium without the radiochemical to chase the label into the high-molecular-weight DNA. The cells were exposed to MC for 60 min in the absence or presence of 1 mM MIX and rinsed twice with ice-cold PBS. The cells were then removed from the dish by trypsinization at 0 °C. Before trypsinization, the cells were irradiated with 8 Gy with a Toshiba KXC-18 X-ray machine (180 kVp, 25 mA, 2.0 mm Al filter, 5 Gy/min), in an ice bath. Approximately 5×10^5 cells in 20 ml phosphate buffered saline (PBS) were loaded onto polycarbonate filters (25 mm diameter, 2.0 μ m pore size, Nucleopore Corp., Pleasanton, CA) and rinsed twice with 10 ml of ice-cold PBS. The cells were then lysed with 5 ml of ice-cold lysis solution (0.2% Sarkosyl, 0.02 M EDTA, 2 M NaCl, pH 10.0) and rinsed once with 5 ml of ice-cold 0.02 M EDTA (pH 10.0). All filtration procedures mentioned above were performed by allowing the solutions to pass through the filters by gravity. DNA was then eluted with an elution buffer (0.1 M tetrapropyl ammonium hydroxide–0.02 M EDTA, pH 12.2) in the dark at a rate of 0.035–0.045 ml/min, using Gilson minipuls 2 at room temperature. Radioactivity in each fraction (3.5–4.0 ml) and that remaining on the filter after elution was counted using a Packard Tricarb 460 CD scintillation counter.

Uptake and Efflux of MC The amount of nonbound MC in cells was examined by the method of Wallner *et al.*¹⁷⁾ The CHO (8×10^6), HeLa (4×10^6) or K-balb (4×10^6) cells exposed to MC for various periods of time with or without MIX were washed extensively with ice-cold PBS, scraped into 1.5 ml of ice-cold distilled water and transferred to a test tube. MC was extracted by adding 3 ml of a 1:1 (v/v) mixture of 2-isopropanol and chloroform and by mixing it well. After centrifugation, the lower organic phase was transferred to another tube and dried out at 40 °C under a stream of nitrogen. MC was redissolved in 50 μ l of methanol. A 20 μ l sample was applied to a Cosmosil 5C₁₈ reverse-phase column (15 cm) and eluted with methanol at 0.5 ml/min, using a Shimadzu LC-6A HPLC system. MC was monitored at 360 nm²³⁾ and the amount was calculated from a standard curve. To assess efflux, the cells incubated with MC for 60 min in the absence or presence of MIX were washed three times with ice-cold PBS. Then, the cells were incubated again with a culture medium pre-warmed at 37 °C and not containing MC, in the absence or presence of MIX, respectively.

Enzyme Assay The DT-diaphorase activity was assayed by the method of Dulhanty and Whitmore.¹³⁾ The enzyme source was prepared by sonicating $1\text{--}5 \times 10^6$ cells in 1 ml of distilled water. The amount of protein in the cell sonicates was determined using a Biorad protein assay kit. For DT-diaphorase assay, 600 μ l of sonicate was added to 2.4 ml of incubation mixture (40 mM Tris–HCl (pH 7.5); 0.7 mg/ml BSA; 0.01% Tween 20; 5 μ M FAD; 200 μ M NADPH; as a final concentration) and preincubated at 37 °C for 3 min. The reaction was started by adding 10 μ l of 12 mM dichloroindophenol in water. The reaction dye was monitored at 600 nm time-dependently at 37 °C with a Shimadzu UV-3000. The reduction without cell sonicates was subtracted from each data. NADPH–cytochrome P450 reductase was assayed according to Phillips and Langdon.²⁴⁾ Six hundred μ l of sonicate prepared as above was added to 2.4 ml of incubation mixture (330 mM phosphate buffer, pH 7.6; 50 μ M NADPH; 1 mM KCN; 50 μ M cytochrome c; as a final concentration). The reaction was started by the addition of 30 μ l of 5 mM NADPH in water. The activity was determined at 30 °C by monitoring the rate of reduction of cytochrome c from the difference in the absorbancy change at 550 nm as an absorption maximum (extinction coefficient of 27.7 mm^{−1} cm^{−1}) and at 539 nm as an isosbestic point, with the Shimadzu UV-3000, dual wavelength mode.

cAMP Assay Exponentially growing CHO cells ($5\text{--}10 \times 10^6$ cells) were treated with 0–100 μ M forskolin or 1 mM MIX for 60 min. After the treatment, the cells were washed three times with ice-cold PBS and fixed with 1 ml of ice-cold 5% trichloroacetic acid (TCA) and scraped into a test tube. The centrifugation was performed at $1500 \times g$ for 5 min and the resulting supernatant was extracted three times with 3 vol of ether on ice to remove the TCA. A trace amount of remaining ether in the supernatant was evaporated at 45 °C, and an appropriate volume was applied to the cAMP assay. The cAMP assay was performed using a Yamasa cAMP assay kit. Protein was determined by a Biorad protein assay kit after the TCA-insoluble fraction was dissolved with 1 N NaOH.

RESULTS

When each cell line was exposed to MC at various doses for 60 min, the survival was decreased dose dependently (Fig. 1, open circles). The presence of 1 mM MIX changed the survival curve of these cell lines differently (Fig. 1, closed circles). The sensitization by MIX obtained in CHO cells is consistent with results reported previously.¹⁸⁾ In other cell lines, MIX contrarily reduced the sensitivity to different extents, except in Balb/c 3T3 cells, in which there was a slight sensitization (Fig. 1).

An alkaline elution assay, described in a previous study,²²⁾ was used to determine crosslinks formed in chromosomal DNA. The crosslinks determined by an alkaline elution assay consisted of DNA interstrand crosslinks and crosslinks between DNA and proteins.²⁵⁾ A typical elution pattern of CHO cells is shown in Fig. 2. The DNA of non-irradiated control cells was retained on a filter during alkaline elution, since there were few DNA strand breaks (open circles). Incubation of cells with 1 mM

Fig. 1. Effect of MIX on the Survival of Cells Exposed to MC

The cells were exposed to MC at various doses in the absence of 1 mM MIX (open circles) or in the presence of MIX (closed circles).

Fig. 2. The Formation of Crosslinks Determined by the Alkaline Elution Method

Most of the DNA molecules from nonirradiated cells were retained on the filter (open circles). The elution of DNA through the filter increased because of breaks in DNA strands, since the cells were irradiated at 8 Gy before elution (closed circles). When the cells were exposed to MC, crosslinks were formed between complementary strands or between DNA and protein, thereby reducing the rate of elution of X-irradiated DNA (open squares). The increase in crosslinks by MIX caused DNA to be eluted more slowly (closed squares).

MIX or 10 μ M MC for 60 min did not affect the elution pattern (data not shown). When 8 Gy was given before loading the cells onto the filter, DNA was eluted through

TABLE I. The Effects of MIX on Crosslink Formation in CHO and K-balb Cells

Cell line	MC (μ M)	MIX	Crosslink factor ^{a)}
CHO	7	—	3.11 ± 0.11 (5) ^{b)}
	7	+	5.22 ± 0.53 (5) ^{c)}
	12	—	5.78 ± 0.61 (5)
K-balb	12	+	9.46 ± 0.71 (5) ^{c)}
	7	—	3.13 ± 0.24 (3)
	7	+	2.79 ± 0.33 (3)

^{a)} The factor was defined as $\log(r_0/r_8)/\log(r_0/r_x)$ (see Text). The larger the factor, the more the crosslinks. ^{b)} Mean $\pm$ S.D. (number of samples). ^{c)} Significantly different from the value in the absence of MIX when the Student's *t* test was employed with the $p=0.05$ level.

the filter because of X-irradiation breaking the DNA into small pieces (closed circles). The crosslinks formed by MC reduced the rate of elution of irradiated DNA to some extent (open squares). Since the exposure of cells to MC in the presence of 1 mM MIX caused more crosslinks, the elution rate slowed (closed squares). For quantification of the number of crosslinks, a crosslink factor was defined as $\log(r_0/r_8)/\log(r_0/r_x)$, where r_0 , r_8 , r_x , respectively, are the fraction of DNA retained on the filter for nonirradiated control, the 8 Gy-irradiated control, and the 8 Gy-irradiated MC-treated cells after a volume of 20 ml had been eluted.²⁵⁾ In Table I, crosslink factors thus obtained are shown. There was a dose-dependent increase in the crosslink factor in CHO cells (compare the factor at 7 μ M with that at 12 μ M). MIX caused a statistically significant increase in crosslinks at each dose (Table I). In K-balb cells, on the other hand, the crosslink factor in the presence of MIX was smaller than that in its absence, although the difference is not statistically significant.

Fig. 3. Effect of MIX on Uptake and Efflux of MC

CHO cells were incubated with (closed circles) or without (open circles) 1 mM MIX for the indicated time. When the cells were incubated with MIX from 30 min before MC exposure, similar results were obtained (open squares for incubation without MIX, closed squares for incubation with MIX). In the study for efflux, the cells incubated with MC for 60 min in the absence or the presence of MIX were washed three times with ice-cold PBS. Then, the cells were incubated again with a culture medium prewarmed at 37°C and not containing MC in the absence or the presence of MIX, respectively. The dotted line was obtained by dividing each data in the presence of MIX by 1.6 (see Text).

The uptake and efflux of nonbound MC are shown in Fig. 3. Doses of MC used (50 μ M) here were much larger than those used anywhere else in the present experiment because of the sensitivity of the assay. The method of Wallner *et al.*¹⁷⁾ was used because radiolabeled MC is not commercially available. However, it should be noted that the difference in dose used in this experiment may have affected the results. The uptake of MC by CHO cells was almost the same in the presence of 1 mM MIX as in the absence of MIX for the first few minutes. Thereafter, the intracellular MC dose in the presence of MIX became higher than that in the absence of MIX. The dose at 60 min in the presence of MIX was about 1.6 times that in the absence of MIX. There is the possibility that MIX had not affected the membrane permeability of MC immediately after the treatment with MIX, but affected it several minutes after, so that the uptake for the first few minutes had not been altered. To test this possibility, MIX was added from 30 min before MC exposure, and similar results were obtained (Fig. 3, open and closed squares). In the efflux study, cells exposed to MC for 60 min were washed three times with ice-cold PBS and incubated with a culture medium at 37°C for various periods of time before determination of the MC dose in the cells. Because the initial intracellular dose of MC in the absence of MIX was 1.6 times smaller than that in the presence of MIX, each value in the presence of MIX shown in Fig. 3 was divided by 1.6, which results in the curve shown by the dotted line. The efflux rate was almost the same in both cases (compare dotted line with open circles). The effects of MIX on intracellular doses of MC in HeLa and K-balb cells are shown in Table II. The cells were exposed to 50 μ M MC for 60 min in the presence or absence of 1 mM MIX. MIX caused a slight but not significant decrease in the dose in each cell line.

The effects of 1 mM MIX on NADPH-cytochrome P450

TABLE II. The Effects of MIX on the Intracellular Dose of MC in HeLa and K-balb Cells after Exposure to 50 μ M MC for 60 min

Cell line	MC dose (ng/10 ⁶ cells)	
	-MIX	+MIX
HeLa	23.3 $\pm$ 1.6 ^{a)}	20.2 $\pm$ 1.8
K-balb	15.3 $\pm$ 1.2	12.8 $\pm$ 1.5

a) Mean $\pm$ S.D. of 3 samples. There was no significant difference between the intracellular doses in the presence or absence of MIX in each cell line when the Student's *t* test was employed with the *p*=0.05 level.

TABLE III. Effects of MIX on the Activity of Enzymes Concerning MC Activation in CHO, HeLa and K-balb Cells

Cell line	Reaction mixture	NADPH-cytochrome P450 reductase (nmol/min/mg protein)	DT-diaphorase (nmol/min/mg protein)
CHO	-MIX	4.83 $\pm$ 0.23 (4) ^{a)}	27.0 $\pm$ 1.9 (4) ^{a)}
	+MIX	4.62 $\pm$ 0.17 (4)	23.9 $\pm$ 3.3 (4)
HeLa	-MIX	8.25 $\pm$ 0.79 (3)	873 $\pm$ 44 (4)
	+MIX	8.45 $\pm$ 0.45 (3)	548 $\pm$ 39 (3) ^{b)}
K-balb	-MIX	44.7 $\pm$ 1.8 (3)	1140 $\pm$ 80 (3)
	+MIX	41.1 $\pm$ 2.0 (3)	660 $\pm$ 38 (3) ^{b)}

a) Mean $\pm$ S.D. (number of samples). b) Significantly different from the value in the absence of MIX when the Student's *t* test was employed with the *p*=0.05 level.

TABLE IV. Effects of MIX or Forskolin on Intracellular cAMP Content and Survival of CHO Cells

Treatment	cAMP (pmol/mg protein)	Survival
—	4.7 $\pm$ 0.2 ^{a)}	0.078 $\pm$ 0.005 ^{b)}
Forskolin (100 μ M)	13.0 $\pm$ 0.4 ^{c)}	0.055 $\pm$ 0.004
MIX (1 mM)	14.2 $\pm$ 0.6 ^{c)}	0.0070 $\pm$ 0.0009

a) Mean $\pm$ S.D. of 3 samples. b) Mean $\pm$ S.D. of 6 samples. c) Significantly different from cAMP content in cells with no treatment when the Student's *t* test was employed with the *p*=0.05 level.

reductase and DT-diaphorase are shown in Table III. MIX had almost no effect on the activity of NADPH-cytochrome P450 reductase in each cell line, whereas it reduced the activity of DT-diaphorase significantly in HeLa and K-balb cells, and caused a slight activity reduction in CHO cells (Table III).

cAMP contents in CHO cells after treatment with forskolin at various doses or 1 mM MIX for 60 min were assayed. cAMP contents were 4.7 $\pm$ 0.2 pmol/mg protein in cells with no treatment and were not changed at 0.01 μ M or 0.1 μ M forskolin. A small increase was seen at 1 μ M and 10 μ M forskolin (*i.e.* 5.4 $\pm$ 0.3 and 6.2 $\pm$ 0.4 pmol/mg protein, respectively). There was a 2.4 times increase at 50 μ M (*i.e.* 11.3 $\pm$ 0.6 pmol/mg protein). The cAMP contents in CHO cells treated with 100 μ M forskolin or 1 mM MIX are shown in Table IV. Both chemicals caused a statistically significant increase in cAMP content. Table IV also shows the effects of 100 μ M forskolin or 1 mM MIX on the sensitivity of CHO cells to MC, to determine whether cAMP mediates the sensitizing effect of MIX. The survival of CHO cells exposed to 8 μ M MC was not much

affected by 100 μ M forskolin, but was significantly affected by 1 mM MIX.

DISCUSSION

In the previous study, we showed that the sensitivity of CHO cells to MC was enhanced when the cells were incubated with MIX before and during MC exposure.¹⁸⁾ In the present study, we showed slightly less but still significant sensitization by the treatment only during MC exposure (Fig. 1). A slight sensitization was also seen with Balb/c 3T3 cells, whereas a reduction in sensitivity to MC was found in other cells. The different responses to MIX may not be due to the origin of cells (CHO and V79 cells were derived from a Chinese hamster but responded differently to MIX) or to the repair of crosslinks (cells derived from Fanconi's anemia lacked repair of crosslinks²⁶⁾ and responded to MIX in a similar fashion as normal human cells did).

It has been shown in cultured cells that sensitivity to MC is altered by various factors. Extracellular low pH may change the activity of DT-diaphorase to increase the sensitivity.¹⁵⁾ Deprivation of oxygen or vitamin C may enhance the reductive activation of MC by NADPH-cytochrome P450 reductase and increases the sensitivity.¹⁴⁾ Changes in the contents of enzymes concerning the bioactivation of MC has been shown to cause altered sensitivity.²⁷⁻²⁹⁾ The increase in the sensitivity by MIX in CHO cells, however, cannot be accounted for by changes in the activity of these enzymes (Table III). On the other hand, we found that the activity of DT-diaphorase was suppressed by MIX in HeLa cells and K-balb cells (Table III). Hence, it is possible that the suppression of the activity of DT-diaphorase may play some role in the reduction of the sensitivity by MIX in these cells. Caffeine, another methylxanthine, is also a potent inhibitor of DT-diaphorase and reduces the sensitivity to MC.³⁰⁾

Permeability is another factor which can change sensitivity. It is reported that heat treatment produces an increase in the permeability of MC which would then cause an increase in the intracellular dose of nonbound MC.¹⁷⁾ In the present study, we showed in CHO cells that an intracellular dose of MC was increased in the presence of MIX from 20 to 60 min after the addition of MC. On the other hand, the intracellular dose of HeLa and K-balb cells whose sensitivity to MC was reduced by MIX was slightly decreased (Table II). The facts strongly suggest that the increase in the MC dose seen with CHO cells may be responsible for the sensitization of sensitivity to MC by MIX.

It is shown in *in vitro* and *in vivo* systems that a DNA monoadduct as well as biadduct (crosslink) is formed³⁻¹⁰⁾ and that a monofunctional MC derivative which cannot form crosslinks had a cell killing effect (data not shown). Active oxygens are also formed by incubation with MC.⁹⁾ Among these lesions, crosslink formation, a major damage caused by MC, is easily quantified by an alkaline elution assay. We showed in Table I that the extent of the crosslinks was increased by MIX treatment in CHO cells, whereas it was decreased slightly in K-balb cells. These changes are well reflected by the changes in the sensitivity

to MC in both cell lines. It is thus probable that an enhanced intracellular dose of MC in CHO cells may cause the increase in crosslink formation and may lead to enhanced cell death. On the other hand, the decreased sensitivity found HeLa and K-balb cells may be due to the suppression of DT-diaphorase and/or to a decrease in MC dose.

MIX is known to be a chemical that raises the cAMP level in mammalian cells. Hence, the cAMP level could be related to the increase in the sensitivity by MIX. In earlier reports,^{31,32)} increased levels of cAMP caused the enhancement of sensitivity to MC and *cis*-diamminedichloroplatinum (II). However, there seems to be no participation of cAMP in the sensitization shown in the present study.

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