KOREAN JOURNAL OF ZOOLOGY
Vol. 18, No.3, 131–140 (1975)

Sensitization Effects of Thymidine Analogs on Methyl Methanesulfonate Induced DNA Repair synthesis

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Methyl Methanesulfonate에 의한 DNA 回復合成에 미치는 Thymidine 相似體의 感受性 效果

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(Received May 14. 1975)

摘 要

HeLa S_3 細胞를 재료로 DNA 回復合成에 미치는 Methyl methanesulfonate (MMS)와 thymidine 相似體 (BUdR, IUdR)의 이중효과를 농도와 시간변화에 따라 ^{3}H-thymidine 처리에 의한 自己放射法으로 조사한 결과는 다음과 같다.

1. MMS를 단독 처리한 경우 標識細胞의 빈도는 MMS의 농도 증가에 따라 증가한다. 이는 DNA 回復合成을 한 細胞의 빈도가 증가한 결과로 細胞當 Grain數의 증가현상과 일치한다. 시간변화에 따른 DNA 回復合成은 MMS와 ^{3}H-thymidine 처리후 2~3시간에 최대증가율을 보인다.

2. BUdR 또는 IUdR의 단독처리는 DNA 回復合成을 일으키지 않는다. 그러나 MMS와 이중 처리할 경우 標識細胞, DNA 回復合成細胞, 기리고 細胞當 Grain數는 MMS 단독 처리한 경우보다 훨씬 증가한다. 따라서 이 두 물질은 MMS에 의한 DNA 回復合成을 효과적으로 증가시키는 感受性物質로 작용함이 판명되었다.

INTRODUCTION

Methyl methanesulfonate (MMS), an alkylating agent, has been reported to produce several biochemical changes in DNA. Of these, some associated with DNA damage and repair include methylation, depurination, single strand breakage and inductions of unscheduled DNA synthesis and repair replication (Strauss and Hill, 1970; Buhl and Regan, 1973). The later two types of DNA repair synthesis are thought to represent the same molecular repair processes of damaged DNA (Regan and Setlow, 1973; Cleaver, 1974). The mechanism by which MMS-induced DNA damage being repaired has been suggested to be a similar to that involved

in the repair of ionizing radiation damage to DNA which is associated with strand breaks (Clarkson and Evans, 1972; Fox and Fox, 1973). Though there has been made of the DNA repair synthesis induced by MMS, quantitative data on the characterization of MMS-induced DNA repair synthesis have scarcely been reported.

It has long been recognized that an incorporation of thymidine analogs, particularly 5-bromodeoxyuridine (BUdR), into DNA of mammalian cells resulted in an increase in their sensitivity to UV-light and X-rays (Djordjevic and Szybalski, 1963). This base analog has been demonstrated to increase the primary lesions in DNA or to influence the repair processes of damaged DNA in irradiated cells following exposure to this compound (Lohman *et al.*, 1972; Sawada and Okada, 1972; Scott *et al.*, 1974).

Up to date, the sensitization effect of this base analog on chemical-induced unscheduled DNA synthesis has not yet been studied. As indicated, DNA damage produced by MMS has been postulated to be repaired by processes similar to those involved in the repair replication by ionizing radiation. If this is correct, it is a matter of some considerable interest to determine whether any thymidine analogs would lead to a cumulative or enhancing effect on MMS-induced DNA repair synthesis.

The present studies were therefore undertaken to characterize the unscheduled DNA synthesis induced by MMS and to determine the sensitization effect of thymidine analogs, BUdR and IUdR, on MMS-induced unscheduled DNA synthesis in HeLa S3 cells.

MATERIALS AND METHODS

1. Cell Culture:

An established human cell line, HeLa S_3 was used throughout this investigation. Monolayer cultures of this cell line were grown at 37°C in milk dilution bottles (Kimax, 120 m*l*) as a stock culture using T.C. medium 199 (Gibco) supplemented with 10% fetal serum and antibiotics (penicillin G, 100 units/m*l*; streptomycin, 100 mcg/m*l*) and maintained in an exponential growth by routine passage using EDTA.

2. Mutagen Treatment:

Methyl methanesulfonate (MMS, Kodak) was dissolved in phosphate buffered saline (PBS) as 1 M stock solution and further diluted to the various working concentrations in the growth medium without serum 10-15 minutes prior to treatment. The sterilization of the growth medium containing MMS was accompanied by passage through a 0.45μ pore size membrane filter inserted into hypodermic adapter. For the induction of unscheduled DNA synthesis, an appropriate number ($0.5—1.0\times10^7$ cells/m*l*) of monolayer cultures grown on cover glasses (9×10 mm) for 48 hours in Leighton culture tubes were treated to MMS from 0

to 2.0 mM for an hour at 37°C. After treatment with MMS, the cells were washed twice with medium and the second washing was replaced with fresh growth medium and incubated.

3. Incorporation of Thymidine Analogs:

5-bromodeoxyuridine (BUdR) and 5-iododeoxyuridine (IUdR) (Sigma) were prepared as 1 M stock solution in growth medium and further diluted to the various working concentrations immediately prior to use. The sensitization effect of these base analogs on MMS-induced unscheduled DNA synthesis was determined by two distinct series of experiments. The first series was conducted to determine the fixed sensitizing effect of BUdR or IUdR on MMS-induced unscheduled DNA synthesis. For this, an appropriate number ($3.0—5.0\times10^6$ cells/m*l*) of cultures grown for 24 hours were exposed to BUdR or IUdR for 24 hours at a final concentration of 0.2 mM prior to the MMS treatment. The second series was performed to determine the dose response of these sensitizers. For this, BUdR or IUdR was added to the cultures at various concentrations (0.016. 0.08 and 0.2 mM) for 24 hours. After treatment with these base analogs, the cultures were washed and treated with MMS as described previously.

4. Unscheduled DNA Synthesis Procedures:

The experiments involving unscheduled DNA synthesis were carried out two different procedures, dose response and time dependence. For the determination of dose response of unscheduled DNA synthesis, ^{3}H-thymidine (Amersham/Searle) was incorporated to the cultures at a final concentration of 10 μC*i*/m*l* (specific activity, 20 C*i*/mM) for an hour immediately after treatment with MMS. For the time dependence study, cells treated with MMS were pulse labeled with ^{3}H-thymidine and then incubated in the medium containing ^{3}H-thymidine up to 4 hours. Labeling with ^{3}H-thymidine was terminated by washing the cells from the cultures three times in cold PBS containing 100 mcg/m*l* of unlabeled thymidine. The cultures grown on cover glasses were directly fixed in 3:1 ethanol glacial acetic acid. Prior to autoradiography, all cover glasses were stained with 2% aceto-orcein and mounted on the slides with the cells uppermost. Autoradiograms were prepared using autoradiographic stripping plate (Kodal AR-10) as described by Park (1975). Silver grains were counted over the nuclei of the labeled cells. Any cell with 5-50 grains above background was considered as an unscheduled DNA synthesizing cell.

RESULTS

The dose response for the MMS-induced unscheduled DNA synthesis in HeLa S_3 cells fixed immediately following treatments with MMS and ^{3}H-thymidine for an hour in each is shown in Table 1.

In the control, 39% of the cells were labeled in which the majority were of

Table 1. Dose response of unscheduled DNA synthesis in HeLa S_3 cells fixed immediately following treatments with MMS and ^{3}H-thymidine labeling.

Treatment MMS (mM)	Labeling[a] index (%)	Labeling pattern (% ±S.E)			Average[c] grains/cell (mean±S.E.)
		Unlabeled	Heavily labeled (S)	Lightly[b] labeled (UDS)	
Control	39.2	60.8±8.1	37.4±3.9	1.8±1.4	5.5±2.2
0.5	50.6	49.4±6.8	36.6±4.0	14.0±3.6	14.2±3.9
1.0	61.6	38.4±4.2	36.2±3.6	25.4±4.7	18.3±4.3
2.0	84.3	15.7±2.9	34.7±3.2	49.6±5.2	27.2±5.1

a: Labeling index was based on the examination of 1,000 cells.
b: Lightly labeled cells were considered as unscheduled DNA synthesizing cells which have less than 50 grains above background in the unclei.
c: Average grains/cell represented the average grain counts/cell (mean±S.E) in all lightly labeled cells in each group.

normal DNA synthesizing cells (heavily labeled). This value was as expected, because the duration of DNA synthetic stage of this cell line is known about 8 hours among T_C=21 hours. The spontaneous unscheduled DNA synthesis (lightly labeled) was also found though the percentage was neglible (1.8%). In the MMS-treated group, the labeling indices were increased in direct proportion to the dose. The labeling pattern indicated that the increased labeling indices were mainly due to increases of lightly labeled cells. The heavily labeled cells were not changed except in the 2.0 mM group. The quantitative analysis of unscheduled DNA synthesis as determined by the average number of grains per cell showed that the grains were increased and directly proportional to dose increased. The increased number of grains was found to be related to the percentage of lightly labeled cells.

From the table it may be cocluded that the dose response of unscheduled DNA synthesis induced by MMS is dose dependent and directly proportional to dose employed, and that the labeling index, percentage of lightly labeled cells and average grains per cell were related.

Table 2 represents the time dependent results of unscheduled DNA synthes is in HeLa S_3 cells fixed at various time intervals following treatments with MMS and ^{3}H-thymidine labeling.

In the control, labeling indices were slightly increased with time. This increase may be due to the accumulation of S-stage cells during the period of prolonged ^{3}H-thymidine incorportation. The percentage of lightly labeled cells and the average number of grains remained unchanged. These may reflect the rate of spontaneous unscheduled DNA synthesis in HeLa S_3 cells under the present experimental conditions. In the MMS-treated group, however, the labeling indices were markedly increased with time in every dose level. Particulary in 2.0 mM group, the labeling indices were virtually 100% at 3 hours and thereafter. The

Table 2. Time dependence of unscheduled DNA synthesis in HeLa S_3 cells fixed at various time intervals following treatments with MMS and ^{3}H-thymidine labelling.

Treatment MMS (mM)	Time* after treatment (h)	Labeling index (%)	Labeilng pattern (%±S.E.)			Average grains/cell (mean± S.E.)
			Unlabeled	Heavily labeled (S)	Lightly labeled (UDS)	
Control	0	40.2	59.8±6.3	38.2±3.5	2.0±1.7	5.5±1.7
	1	42.9	57.1±5.9	40.2±4.7	2.7±1.6	4.6±1.5
	2	47.6	52.4±5.7	45.7±4.9	1.9±1.5	7.0±2.0
	3	49.5	50.5±4.9	47.2±4.2	2.3±1.5	3.0±1.0
	4	52.8	47.2±5.2	50.3±5.3	2.5±1.8	5.5±1.0
0.5	0	49.8	50.2±5.0	37.1±4.1	12.7±2.8	14.2±2.0
	1	55.6	44.4±4.2	39.8±3.9	15.8±2.3	17.3±2.1
	2	64.9	35.1±3.8	41.5±4.5	23.4±2.8	20.9±1.9
	3	70.4	29.6±3.0	42.9±4.2	27.5±3.5	21.8±2.5
	4	76.0	24.0±3.6	43.5±4.9	27.5±2.9	19.8±1.8
1.0	0	62.6	37.4±2.7	36.0±3.8	26.6±2.7	16.3±2.8
	1	75.1	24.9±3.4	39.9±3.7	35.2±3.5	23.7±3.0
	2	91.8	8.2±2.9	44.6±4.3	47.2±4.9	28.6±3.5
	3	91.6	8.4±2.8	45.3±4.0	46.3±4.8	30.7±3.6
	4	92.2	7.8±1.7	46.2±4.1	46.0±4.7	29.7±2.9
2.0	0	82.8	17.2±2.1	33.7±3.8	49.1±5.2	27.2±3.4
	1	86.5	13.5±1.9	32.6±3.5	53.9±6.3	32.2±3.5
	2	96.3	3.7±1.4	35.2±3.0	61.1±7.5	36.6±4.2
	3	100.0	—	36.2±3.9	63.8±6.9	35.7±3.6
	4	100.0	—	37.7±3.8	62.3±6.4	34.2±3.7

*: 0 hour=immediately after treatment with MMS and ^{3}H-thymidine labeling.

labeling pattern showed that heavily labeled cells were not increased as much as the control, whereas lightly labeled cells were markedly increased in each group. The peak of lightly labeled cells appeared between 2—3 hours after MMS treatment. The average number of grains was also increased with peak appearing at 3 hours in 0.5 and 1.0 mM groups and at 2 hours in 2.0 mM treated group, respectively.

The overall results suggest that unscheduled DNA synthesis induced by MMS is dose dependent and continue up to 4 hours, and that the highest rate of repair processes varies but largely appears at 2~3 hours after treatment with MMS.

Table 3 shows the effect of 0.2 mM BUdR or IUdR on MMS-induced unscheduled DNA synthesis in HeLa S_3 cells fixed at I hour following treatment with MMS and ^{3}H-thymidine labeling.

As shown in the table, the single treatment of these thymidine analogs does not induce unscheduled DNA synthesis. However, in the combined treatment with BUdR+MMS or IUdR+MMS, the labeling indices, percentages of lightly labeled cells and average grains per cell were markedly increased as dose increased. About 20% of labeling indices were increased in BUdR or IUdR+0.5 and 1.0 mM

Table 3. Effect of BUdR or IUdR on MMS-induced unscheduled DNA synthesis in HeLa S_3 cells fixed at I hour following treatments with MMS and ^{3}H-thymidine labeling.

Treatments BUdR or IUdR (mM)	Treatments MMS (mM)	Labeling index (%)	Labeling pattern (%±S.E.) Unlabeled	Hoavily labeled (S)	Lightly labeled (UDS)	Averago grains/cell (mean±S.E.)
—	—	38.6	61.4±9.2	37.3±4.9	1.3±1.2	5.0±1.9
BUdR 0.2	—	39.4	60.6±8.7	37.4±4.3	2.0±1.7	6.2±2.6
BUdR 0.2	0.5	69.2	30.8±5.0	31.6±4.7	37.6±4.7	20.1±1.5
BUdR 0.2	1.0	81.1	18.9±4.9	30.9±3.6	50.2±6.2	27.3±1.3
BUdR 0.2	2.0	90.4	9.6±3.3	28.0±4.7	62.4±7.3	33.9±3.5
IUdR 0.2	—	39.2	60.8±9.2	36.6±4.2	2.6±1.5	6.5±2.2
IUdR 0.2	0.5	69.5	30.5±6.9	29.1±5.3	40.4±5.2	29.2±1.6
IUdR 0.2	1.0	81.7	18.3±4.5	26.5±4.9	55.2±5.6	35.5±0.9
IUdR 0.2	2.0	96.8	3.2±2.7	24.2±3.7	72.6±7.2	42.6±2.1

MMS treated groups as compared to the corresponding net MMS treated groups. But these values were slightly declined in 2.0 mM group (6% in BUdR+MMS and 12% in IUdR+MMS). The increased percentages of lightly labeled cells were 23.6, 24.8 and 12.8 in BUdR and 26.4, 29.8 and 23.0 in IUdR pretreated groups as compared to the net MMS treated groups in 0.5, 1.0 and 2.0 mM dose levels, respectively. The increased number of grains per cell were 5.9, 9.0 and 6.7 in BUdR+MMS and 15.5, 17.2 and 15.4 in IUdR+MMS treated groups as compared to the corresponding 0.5, 1.0 and 2.0 mM MMS single treatment groups.

The above results clearly indicate that BUdR and IUdR are both potent chemical sensitizers enhancing MMS-induced unscheduled DNA synthesis and IUdR is found to be more effective than BUdR as sensitizer.

The tendency for increased grains in these three experimental groups is illustrated in Fig. 1.

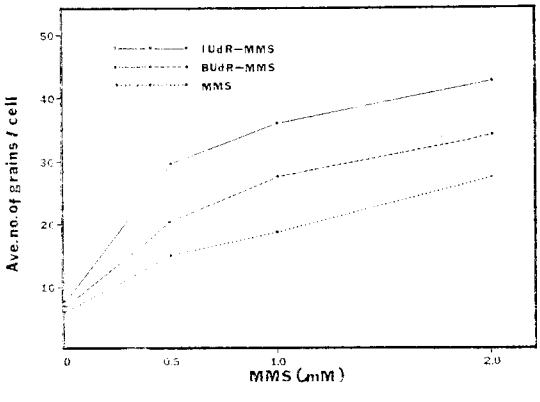

Fig. 1. Sensitization effect of UBdR or IUdR on induced unscheduled DNA synthesis n HeLa S_3 cells.

As shown in the table 1, 3 and Fig. 1, grains in the MMS group were increased in direct proportion to the dose, but BUdR+MMS and IUdR+MMS groups showed an initial sharp increase in grain counts which then slowed as the dose increased. These results might suggest that the combined action on unscheduled DNA synthesis is rate limiting or somewhat inhibiting at the higher dose levels. The data also indicate that the combined action of thymidine analogs and MMS on unsched-

Table 4. Dose response of BUdR or IUdR on MMS-induced unscheduled DNA synthesis in HeLa S3 cells fixed at I hour following treatment with MMS and 3-H-thymidine labelling.

Treatments BUdR or IUdR (mM)	MMS (mM)	Labeling index (%)	Average grains /cell (mean±S.E.)	Fraction of increased grains compared to MMS alone treament (%)
Control	2.0	84.3	27.2±5.1	—
BUdR 0.016	2.0	88.7	30.3±5.5	3.1
BUdR 0.080	2.0	91.2	33.3±5.7	6.1
BUdR 0.200	2.0	90.4	32.9±3.5	4.7
IUdR 0.016	2.0	99.3	40.2±3.5	13.0
IUdR 0.080	2.0	99.0	42.0±3.9	14.8
IUdR 0.200	2.0	96.8	42.6±2.1	15.4

uled DNA synthesis varies at different dose level, and that 1.0 mM MMS is the most effective dose in enhancing MMS-induced unscheduled DNA synthesis.

Table 4 summaries the dose response of BUdR and IUdR on 2.0 mM MMS-induced unscheduled DNA synthesis.

The maximum effective dose in enhancing MMS-induced unscheduled DNA synthesis was different in the two experimental groups. In the BUdR+MMS group, 0.08 mM BUdR induced the highest valus in both labeling index and grain counts, whereas in the IUdR+MMS no marked changes in the labeling index occurred in the first two dose levels and at the higher dose the labeling index was decreased. This result may be due to the decreased rate of heavily labeled cells and not of lightly labeled cells.

From the table it may be concluded that IUdR is more effective sensitizer in MMS-induced unscheduled DNA synthesis. However, the highest effective dose is different in different base analogs, and the dose response of these sensitizers on MMS-induced unscheduled DNA synthesis is not proportional to the dosage increased

DISCUSSION

The repair processes of damaged DNA induced by chemicals are largely divided into two groups, UV-type and ionizing-type repairs. The former is characterized by an insertion of large amounts of new bases (100 nucelotides/dimer) to repair DNA and the latter required only few nucleotides (3-4 nucleotides/strand break) (Regan and Setlow, 1973). MMS has been appeared to be a typical ionizing-type mutagen (Clarkson and Evans, 1972; Fox and Fox, 1973; Sasaki, 1973). A molecular mechanism for the repair of damaged DNA produced by MMS has been postulated that MMS first methylates DNA, primarily on the N-7 position of the guanine and N-3 of the adenine bases, and then the DNA undergoes depurination and single strand breakage, which after further nuclease, polymerase and ligase

activities results in resynthesis of new bases in a non-semiconservative manner (Fox and Fox, 1973).

The dose response for unscheduled DNA synthesis induced by MMS has been reported by several workers (Hahn *et al.*, 1968; Sanes and Okun, 1972; Fox and Fox, 1973). The published data indicate that MMS-induced unscheduled DNA synthesis is dose dependent up to 2.0~3.0 mM, representing about 20~25% lightly labeled cells/mM MMS/h treatment. Clarkson and Evans (1972) reported that incorporation of ^{3}H-thymidine in human lymphocytes after different dose levels was found to increase with increasing dosage up to 3.0 mM and then it declined at higher concentrations. This result is also consistent with that of autoradiography as indicated in this paper. The quantitative analysis of grain counts was only reported by Fox and Fox (1973), who showed that about 25 grains were observed over the lightly labeled cells fixed at 1 hour after treatments with MMS and ^{3}H-thymidine labeling. The present results are in good accord with those of Fox and Fox (1973).

The labeling index has been suggested to be influenced by concentration and specific activity of labeled nucleotide and by exposing time of autoradiogram (Cleaver, 1971). In addition, it can not be ruled out the factors of T_G and stages of cell cycle. Relatively high percentage of lightly labeled cells occurred in the present results may be explained due to these factors.

Studies on the time dependence of unscheduled DNA synthesis are used as a parameter for determiningg the repair processes of induced DNA damage. Cleaver (1971) first reported that repair replication was continued up to 5 hours in human fibroblast and HeLa cells treated with 0.1 mM MMS, whereas in higher dose levels (0.5 and 1.0 mM) it was completed within an hour. Clarkson and Evans (1972) subsequently reported that the rate of ^{3}H-thymidine incorporation in human lymphocytes after treatment with 1.0~3.0 mM MMS was the highest during the first hour and then the rates decreased but continued for as long as 21 hours after treatment. Fox and Fox (1973) reported that the portion of heavily labeled cells remained unchanged but there was an increase in the lightly labeled cells with time resulting in virtually 100% labeling by 6 hours, and that grain counts were also increased with time at a similar rate.

The results presented here concerning the time dependence of unscheduled DNA synthesis induced by MMS are in good agreement with those of Fox and Fox (1973) in both labeling index and grain counts, as well as in good accord with those of Clarkson and Evans (1972). It was suggested that the initial fast reaction after ionizing radiation and MMS might involve the incorporation of only one or two nucleotides per lesions (Clarkson and Evans, 1972). From the point of view, it is tempting to conclude that the fast reaction processes might be involved in the

rejoining of single strand breaks in the DNA and that the slow processes may be associated with damage other than single strand breaks (Fox and Fox, 1973).

It has been demonstrated that the halogenated thymidine analogs increase the radiosensitivity of mammalian cells by virtue of their incorporation into DNA in place of thymine and influence DNA repair synthesis induced by radiation (Djordjevic and Szybalski, 1963; Rasmussen and Painter, 1966; Sawade and Okada, 1972). So far no one has attempted to study the sensitizing action of BUdR or IUdR on chemical-induced unscheduled DNA synthesis. Therefore, there are no available data to compare with the results reported here.

It is postulated that an incorporation of base analogs into the DNA in the place of thymine enhances an increase of the primary lesions in DNA leading to single strand breaks attributed to a random increase in energy absorption of the BU-containing DNA strand after mutagen treatment, which can be repaired by repair replication or other repair processes such as single strand rejoining (Lohman *et al.*, 1972; Sawada and Okada, 1972). The present experimental results clearly demonstrate that BUdR and IUdR are both potent sensitizers enhancing unscheduled DNA synthesis. However, it should be explained how these two different chemicals act on DNA molecules as mutagen to enhance unscheduled DNA synthesis. One of the possible explanations on these results is that the increased amount of purine bases due to the transition mutation may provide a large proportions of action sites to be attacked by MMS (Legator and Flamm, 1973). Further studies are necessary to determine the molecular mechanism for the sensitization action of thymidine analogs on chemical induced DNA repair synthesis.

SUMMARY

Dose response for the unscheduled DNA synthesis induced by various concentration of MMS was dose dependent and directly proportional to dose increased. Time dependence of unscheduled DNA synthesis was continued up to 4 hours, with the peak appearing 2~3 hours after treatment with MMS and ^{3}H-thymidine labeling. Single treatment with BUdR or IUdR does not induce unscheduled DNA synthesis. BUdR and IUdR greatly enhanced MMS-induced unscheduled DNA synthesis, but the dose responses were different from that of single treatment with MMS. IUdR was found to be more effective sensitizer on MMS-induced unscheduled DNA synthesis.

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