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THE INHIBITION OF DNA REPAIR IN PRIMARY RAT HEPATOCYTE CULTURES BY APHIDICOLIN: EVIDENCE FOR THE INVOLVEMENT OF ALPHA POLYMERASE IN THE REPAIR PROCESS

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SUMMARY: The effect of aphidicolin on the repair of chemically induced DNA damage in rat hepatocytes was examined. Alkaline elution analysis of DNA damage and autoradiographic examination of unscheduled DNA synthesis both indicate that the repair of DNA damage was inhibited by aphidicolin. Because aphidicolin has been shown to be a specific inhibitor of alpha polymerase, these results suggest that the alpha polymerase plays an active role in the repair of rat hepatocyte DNA.

Alpha polymerase has been implicated as the principal polymerase mediating the replication of cellular DNA in eucaryotic cells (1-4) whereas the beta polymerase is thought to mediate DNA repair (5-8). The fungal antibiotic aphidicolin has been shown to specifically inhibit alpha polymerase and thus replicative DNA synthesis. Whether the specific inhibition of alpha polymerase affects the repair of DNA has been examined and various conclusions have been reached. Inhibition (9-12), lack of inhibition (10,12-14) and partial inhibition (15) of DNA repair by aphidicolin has been reported. These conflicting results as to the role of alpha polymerase in mammalian cells may be partially due to the variety of systems (eg.whole cells, permeabilized cells, nuclear preparations) and cell types used (eg. normal, transformed, human, rodent) or may indicate that the polymerase is involved to different extents in different cell types.

Primary hepatocyte cultures have been utilized by several groups to assess the genotoxicity of various chemicals using the induction of DNA repair as an endpoint (16-18). It is therefore of interest to understand the mechanism that mediates the DNA repair process in these cells. This study examines

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the effect of aphidicolin on the induction of DNA damage and DNA repair by MMS[1] in whole, normal hepatocytes isolated from adult rats. The results presented here support the hypothesis that alpha polymerase is actively involved in the replication and repair of hepatocyte DNA.

MATERIALS AND METHODS:

Cell isolation and culture:

Single cell suspensions of rat hepatocytes were prepared by collagenase perfusion of adult male Fischer-344 rats (COBS CD F/Crl BR, Charles River Breeding Labs, Kingston, NY) as described by Williams et al. (19). The preparation of primary hepatocyte cultures was performed as previously described (20).

Cell treatment:

Fresh stock solutions of aphidicolin were prepared in dimethylsulfoxide (final concentration did not exceed 0.2%) and diluted to the appropriate concentrations (0,1,3, or 6 μg/ml) with WEI (Flow Labs). Subsequent to a 1 hr incubation with aphidicolin, the cultures were treated with 1.2 or 0.6 mM MMS (Aldrich) for 0.5 hr, by dilution of stock MMS in the plates containing aphidicolin and WEI.

Alkaline elution:

The alkaline elution procedure used in this study was that described by Kohn (21) as modified for the assessment of DNA damage in nonlabeled cells (20). The induction of DNA damage by MMS was examined immediately following treatment or after an additional 2 hr incubation in the absence of MMS.

Unscheduled DNA synthesis:

Cell cultures were processed for autoradiographic quantitation of UDS as previously described (17). Following the treatment interval, the cells attached to coverslips were washed three times with WEI, incubated in 2 ml WEI containing 10 μCi/ml [^{3}H]thymidine (42 Ci/mmol, Amersham Corp.) and 0,1,3, or 6 μg/ml aphidicolin for 2 hr, washed again with WEI, fed WEI supplemented with 5% fetal calf serum and incubated overnight. Where the number of cells in S-phase was to be determined the cells were washed and fixed immediately after the 2 hr incubation with [^{3}H]-thymidine. Fixed cell preparations were dipped in NTB2 photographic emulsion (Kodak), exposed for 10 days and scored as previously described (17).

RESULTS:

Effect of aphidicolin on replicative DNA synthesis of hepatocytes.

Primary cultures of hepatocytes generally have a small fraction (0.1 to 1.0%) of the population that is actively synthesizing DNA. The sensitivity of this replicative DNA synthesis to inhibition by aphidicolin was confirmed by

[1]Abbreviations: MMS, methylmethanesufonate; WEI, Williams medium E supplemented with 2 mM glutamine; UDS, unscheduled DNA synthesis.

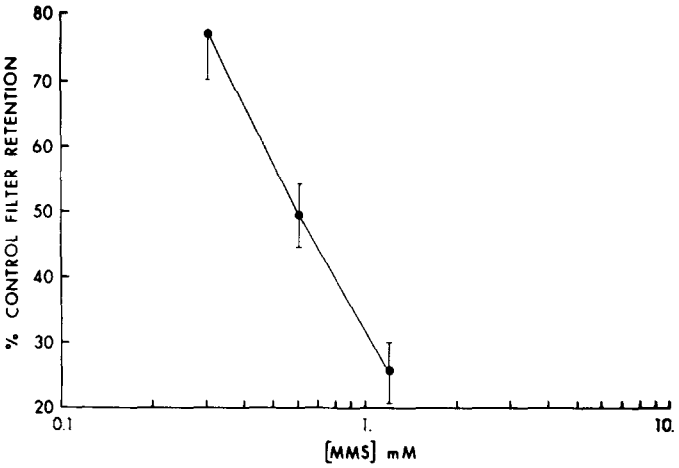

Figure 1. Alkaline elution of rat hepatocytes treated in vitro with methyl methanesulfonate. Each point represents the mean of at least three experiments ± the standard error of the mean.

autoradiographic examination of the cell preparations. Cells in S-phase were readily distinguished from those in repair by the high density of silver grains over the nuclei in S-phase. Reductions of 76 ± 6% in the number of cells in S-phase were observed when these cells were exposed to 6 μg/ml aphidicolin. Further reductions, however, were not observed following treatment with 20 μg/ml (data not shown).

Effect of aphidicolin on MMS induction of DNA damage in hepatocytes.

Cultures treated with 3 μg/ml aphidicolin were examined for the presence of DNA damage with no difference from control cells being observed (Table I). In contrast, alkaline elution analysis of DNA from MMS treated hepatocytes revealed that DNA damage increased linearly with dose (Figure 1). Incubation of MMS treated cells, in the absence of MMS, for an additional 2 hr resulted in greater filter retention of DNA than when the cells were not incubated (Table I).

The presence of 3 μg/ml aphidicolin during the 0.5 hr exposure to MMS resulted in less DNA being retained than when MMS alone was used. Similarly, 2 hr post-treatment incubation of MMS treated hepatocytes with aphidicolin prevented the increase in DNA retention observed when aphidicolin was absent (Table I).

TABLE I

The effect of aphidicolin on the induction and disappearance of DNA damage in hepatocytes treated with MMS

Hours post-treatment	% of control retention[a]		
	Hepatocytes +		
	Aphidicolin	MMS	Aphidicolin, MMS[b]
0	92 ± 1.7[c]	44 ± 6.4	27 ± 3.0
2	94 ± 1.5	81 ± 4.2	34 ± 8.3

[a]Retention of control DNA was 97 ± 2.8%

[b]Freshly isolated hepatocytes were treated with 3 μg/ml aphidicolin for 1 hour, with 0.6 mM MMS for 0.5 hour or with both.

[c]Standard error of the mean of three experiments.

Effect of aphidicolin on MMS induced UDS in hepatocytes.

Control cells and cells treated with 1,3, and 6 μg/ml aphidicolin had no measureable differences in the number of grains over the nucleus or in the % of the population in repair (Table II). Hepatocyte cultures treated with 1.2 and 0.6 mM MMS were examined autoradiographically for the induction of UDS with and without aphidicolin present. Treatment with MMS resulted in an elevation in net grains per nucleus observed over controls, whereas, inclusion of aphidicolin during treatment with MMS and during incorporation of [^{3}H]thymidine resulted in substantial reductions in the net grains per nucleus observed (Table II).

DISCUSSION:

Several lines of evidence have implicated alpha polymerase as the principal enzyme mediating the replication of DNA in mammalian cells (6,2,3,25), particularly the specific inhibition of the enzyme by aphidicolin (1,26). This idea is supported by the sensitivity of freshly isolated hepatocytes to the inhibition of DNA synthesis by aphidicolin as measured by the reduction in the number of cells in S-phase. The extent of inhibition, though substantial, is not as great as has been reported for other cells (4,8,12) and this may be

TABLE II

The effect of aphidicolin on MMS induced UDS in freshly isolated hepatocytes[a]

	Aphidicolin (μg/ml)							
	0		1		3		6	
[MMS] (mM)	Net[b] grains	% in repair[c]	Net grains	% in repair	Net grains	% in repair	Net grains	% in repair
0	-3.5 ± 1.3	1.7 ± 0.8	-2.1 ± 1.0	0.7 ± 0.6	-2.3 ± 0.2	3.5 ± 3.5	-4.1 ± 1.6	0 ± 0
0.6	10.5 ± 2.4	73.8 ± 7.2	4.2 ± 3.5	35.8 ± 15.2	3.6 ± 2.7	25.7 ± 20.0	1.6 ± 0.7	18.8 ± 5.4
1.2	17.4 ± 5.4	86.0 ± 8.4	4.5 ± 0.9	38.7 ± 12.0	3.7 ± 1.4	28.6 ± 15.0	4.3 ± 1.6	41.9 ± 13.0

[a] Freshly isolated hepatocytes were incubated for 1 hr with 1, 3, or 6 μg/ml aphidicolin followed by exposure to 0.6 or 1.2 mM MMS for 0.5 hr. All cultures were incubated for 2 hrs after treatment with 10 μCi/ml [^{3}H]-thymidine, followed by overnight incubation in the absence of [^{3}H]-thymidine.

[b] The mean net grains/nucleus ± the standard error of the mean for at least two separate experiments.

[c] % in repair is defined as the fraction of the population with 5 or more net grains/nucleus. The data represents the mean ± the standard error of the mean for at least two separate experiments.

partially attributable to an escape of part of the population from the block, due to the metabolism of aphidicolin in these cells (22). A role for beta polymerase in the semiconservative replication of DNA is not excluded by these findings.

Primary cultures that had been exposed to MMS were shown to have sustained DNA damage that was detectable by alkaline elution (Figure 1). Further incubation of these cells, in the absence of MMS, resulted in a reduction of the quantity of DNA damage detectable (Table I). Presumably, the observed post-treatment reduction in damage was due to the repair of the DNA and this is consistent with the induction of UDS by MMS in the same cell population (Table II).

Treatment of primary hepatocyte cultures with aphidicolin revealed that aphidicolin did not induce DNA damage (Table I). Since rat liver microsomes are capable of metabolizing aphidicolin (22) these results also suggest that the metabolites produced are also not able to induce DNA damage in whole hepatocytes. The presence of aphidicolin during MMS treatment or post-treatment incubation resulted in an accumulation of DNA breaks, consistent with the work of Waters (23) and with the inhibition of DNA repair observed by others (8-11, 24). Further evidence for the inhibition of DNA repair was provided by the aphidicolin mediated reduction in MMS-induced UDS. Although substantial inhibition of UDS by aphidicolin was observed there was a resistant component which may be ascribable to beta polymerase activity. These results are in contrast to those of Seki et al. who reported that aphidicolin did not inhibit bleomycin-induced DNA repair in rat liver nuclei (24) and some permeable rodent cells (15). These conflicting results may be due to the difference between whole cells, nuclei and permeable cells or to a difference in the mechanism operative in the repair of different types of damage (i.e. methylated purines and pyrimidines versus strand breaks).

In conclusion, the results presented here suggest that alpha polymerase is operative not only in the semiconservative replication of DNA but in the repair of chemically damaged DNA of rat hepatocytes.

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