CAFFEINE-SENSITIVE REPAIR OF ULTRAVIOLET LIGHT-DAMAGED DNA OF MOUSE L CELLS

Yoshisada Fujiwara and Teruo Kondo

Laboratory of Medical Biophysics, Kobe University
School of Medicine, Kobe 650, Japan

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SUMMARY Mouse L5 cells, although as resistant to UV as excision-capable HeLa S3 cells, do not excise thymine dimers yielded at a rate of 0.05% thymine per 100 ergs/mm^2. The daughter DNA newly replicated for 2 hours in 200 ergs/mm^2-irradiated L5 cells is discontinuous with gaps spaced at about 5 X 10^6 daltons and seemingly complementary to photoproduct on the parental strand. The gapped DNA is subsequently sealed by the postreplication repair. Caffeine (2mM) inhibits such a DNA repair in irradiated L5 cells, but not in HeLa S3 cells. This result is correlated with caffeine-effected depression of recovery in the colony-forming ability of L5 cells after irradiation.

The Pettijohn and Hanawalt method (12) can detect more or less repair replication in all types of mammalian cells (3, 4). However, the excision of pyrimidine dimers in rodent cells has not been demonstrated (9, 10, 15). A growing evidence (5, 6, 11, 13) indicates that cells of rodent origin possess some mechanism for the bypass of the photoproduct of DNA by which the cells recover through DNA synthesis after UV-irradiation. Rupp and Howard-Flanders (14) have demonstrated recombinational repair, i. e., the postreplication repair, in an excision-defective strain of E. coli.

Rauth and Domon (6, 13) have also shown that caffeine reduces UV-survival of mouse L cells by inhibiting the recovery requiring DNA synthesis. However, caffeine exerts no specific effect on repair replication in HeLa cells which possess the ability of excision repair (4).

This paper describes effects of caffeine on the postreplication sealing of UV-induced daughter-DNA gaps in mouse L cells which is assumed to be a process similar to that proposed for excision-defective bacteria (14).

MATERIALS AND METHODS Mouse L5 cells derived from Earle's L929 cells and

HeLa S3 cells [generously provided by Dr. T. Terasima, National Institute of Radiological Sciences, Chiba, Japan] were cultured in F10 medium as described previously (7) and synchronized either by harvesting cells in mitosis (7) or by induction for 16 hours with 1 μM 5-flurodeoxy-2'-deoxyuridine (FUdR) (8).

Either synchronized or random cells were exposed to UV (254 nm) at an incidence dose of 7.3 ergs/mm^2/sec, as determined by inactivation of T4D phage [kindly supplied by Dr. S. Kondo, Osaka Univ., Osaka, Japan]. Immediately or at various lengths of time after UV irradiation, 1 or 2 mM caffeine (Sigma) was added to the cultures for the assay of colony-forming ability and analysis of DNA sedimentation characteristics in alkaline sucrose gradients.

For the latter, cells which had been prelabeled uniformly with 0.2 μCi/ml ^{14}C-thymidine (TdR) (30 mCi/mmole, Schwarz BioResearch) for 2 days were partially synchronized with 1 μM FUdR for 16 hours and exposed to 200 ergs/mm^2 at the late G1 window. Their DNA synthesis was reversed with 25 μCi/ml TdR-methyl-^{3}H (25 Ci/mmole, The Radiochemical Centre) for 2 or 24 hours in the presence or absence of caffeine. Approximately 2.5 X 10^4 labeled cells were lysed in 0.5 ml of 0.25% SDS -0.015M EDTA-0.15M bicarbonate, pH 8.0, and 0.4 ml of 5 mg/ml preheated pronase (Kaken Chemicals, Tokyo) was added. The lysate was digested for 4 hours at 37°C. After addition of 0.1 ml 3N NaOH, 0.2 ml out of the final 1.0 ml lysate, which corresponds to 5 X 10^3 cells (less than 0.1 μg denatured DNA), was overlaid on top of the 4.8 ml linear gradient of 5%-20% sucrose solution containing 0.8M NaCl, 0.2M NaOH, 0.01M EDTA and 0.025M 4-aminosalicylate, pH 12.5. The gradients were centrifuged for 2 hours at 35,000 rpm in a Spinco SW39L rotor (Beckman Instruments at 20°C. Weight and number average molecular weights (Mw and Mn, respectively) were calculated by the formulas of Abelson and Thomas (1) and Charlesby (2) using labeled T4D DNA (the denatured form: 66 X 10^6 daltons) for reference.

The detection of thymine dimers was carried out according to the method of Setlow et al. (16). The radioactivity of acid-insoluble DNA collected on filters (GSWP, Millipore) and dimers on the chromatographed paper strips was assayed in PPO-POPOP-toluene.

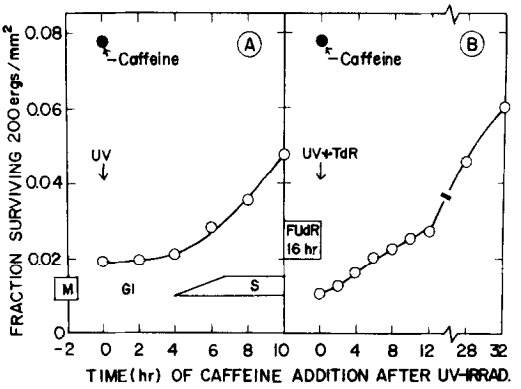

Figure 1. Recovery of synchronized L5 cells from UV damage as revealed by delayed addition of 1 mM caffeine. (A) Cells in mitosis were harvested at -2 hour and exposed to 200 ergs/mm^2 at 0 hour. (B) FUdR-synchronized late G1 cells were irradiated and rescued with 10 μM TdR. 1 mM caffeine was added at the times indicated and continuously present until colonies developed. •; control survival in the absence of caffeine, o; survival of cells treated with caffeine. Plating efficiency of caffeine-treated cells was over 90% of the untreated control.

RESULTS Table 1 shows distributions of ^{3}H-radioactivity between thymine (T) and thymine dimers ($\widehat{TT}$). $\widehat{TT}$ is yielded at an approximate rate of 0.05% T/100 ergs/mm^2 in both L5 and HeLa S3 cells. L5 cells cannot excise $\widehat{TT}$ even at 24 hours after irradiation with various UV doses, as compared with the excision of more than 50% in HeLa S3 cells. This result is agreed with other observations (9, 10, 15).

Subsequebtly, it is essential for studying the bypass of the photoproduct to test whether or not our L5 cells carry out caffeine-sensitive recovery in colony survival which requires DNA synthesis, as Rauth (13) has demonstrated. Early G1 cells at 2 hours after harvesting mitotic cells (Fig. 1A) and late G1 cells induced with FUdR for 16 hours (Fig. 1B) were exposed to a single dose of 200 ergs/mm^2. Delayed addition of 1 mM caffeine at the times indicated was followed to see to what extent the irradiated cells recover as a function of time until addition.

The surviving fraction is around 0.08 in both early (Fig. 1A) and late G1 cells (Fig. 1B) in the absence of caffeine. Caffeine (1 mM) added immediately after irradiation reduces survival to approximately 0.02 (Fig. 1A and 1B), showing that caffeine depresses recovery, which is specifically connected with irradiated

cells since the same amount of caffeine exerted no marked toxicity on unirradiated cells (more than 90% of the control plating efficiency). However, upon delayed addition, the cells recover as they progress only through the postirradiation S period (Fig. 1A and 1B), but not the G1 period (Fig. 1A). This result indicating that caffeine-sensitive recovery from UV damage of L5 cells which keep dimers in their DNA (Table 1) requires DNA synthesis confirms the previous finding with mouse L cells of Rauth and Domon (6, 13). This characteristic recovery operates efficiently in L5 cells, since the 1/e dose (=105 ergs/mm^2) for them is almost

Table 1. Distributions of Radioactivity Between Thymine (T) and Thymine Dimer ($\widehat{TT}$) in DNA from UV-irradiated Mouse L5 and HeLa S3 Cells#

UV doses (ergs/mm^2)	Hours after UV-irradiat.	Counts/minute		$\widehat{TT}$/T(%)
		T	$\widehat{TT}$	
Mouse L5 cells				
0	0	336,000	19	0.006
400	0	335,000	692	0.204
400	24	159,000	319	0.201
750	0	221,000	831	0.375
750	24	216,000	830	0.383
1,000	0	268,000	1,470	0.558
1,000	24	183,000	1,016	0.552
HeLa S3 cells				
0	0	314,000	19	0.006
1,000	0	664,000	3,018	0.455
1,000	24	132,000	330	0.250

More than 5 X 10^7 cells were prelabeled with 2 μCi/ml ^{3}H-TdR for 24 hours and chased for several hours with 50 μM unlabeled TdR. At 0 and 24 hours after irradiation, DNA was extracted, hydrolyzed with formic acid and chromatographed on Whatman No. 1 paper for 12-16 hours using the solvent of n-BuOH-glacial acetic acid-H_2O (80:12:30). The radioactivity of the paper strips (1 X 2.5 cm) was assayed in PPO-POPOP-toluene.

the same as for HeLa S3 cells (unpublished data).

In order to study the molecular mechanism of caffeine effect on the bypass, especially in relation to DNA synthesis, ^{14}C-TdR-prelabeled L5 cells were partially synchronized with 1 μM FUdR for 16 hours and exposed to either 0 or 200 ergs/mm^2. DNA synthesis of the cultures was immediately reversed with 25

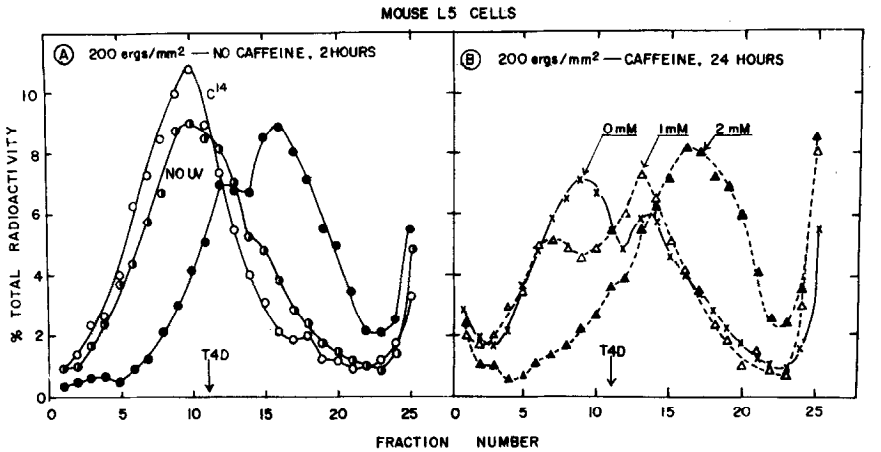

Figure 2. Alkaline sucrose sedimentation profiles of the DNA from L5 cells that were prelabeled with ^{14}C-TdR, partially synchronized with 1 μM FUdR for 16 hours and irradiated with 200 ergs/mm^2 at the late G1 window. DNA synthesis was reversed immediately with 25 μCi/ml ^{3}H-TdR for 2 (A) and 24 hours (B). The ^{3}H-labels are: ○; no UV, no caffeine, 2 hours (unirradiated control), ●; UV, no caffeine, 2 hours, x; UV, no caffeine, 24 hours, Δ; UV, 1 mM caffeine, 24 hours, ▲; UV, 2 mM caffeine, 24 hours. Only single profile of the parental ^{14}C-DNA (o) from unirradiated cells is depicted in A, since it did not change significantly between variously treated cells. Arrow indicates the position at which intact T4D DNA sediments under the identical condition.

μCi/ml ^{3}H-TdR with or without caffeine. The DNA from double-labeled cells was centrifuged in alkaline sucrose gradients.

Fig. 2A presents the profiles of DNA labeled for 2 hours. The ^{3}H-DNA normally replicated after no UV irradiation (Fig. 2A; semiopen circle) sediments nearly as fast as the ^{14}C-template (Fig. 2A; open circle), Mw of which is about 100 X 10^6 daltons. The ^{14}C-parental DNA in irradiated cells sediments exactly at the same position as the unirradiated control (Fig. 2A; open circle), indicating that the parental DNA of L5 cells is not broken by excision. However, ^{3}H-daughter DNA in irradiated cells (Fig. 2A; closed circle) sediments more slowly, Mn (= 1/2 Mw) of which is estimated to be about 5 X 10^6 daltons. This corresponds roughly to the average interdimer length of 1 X 10^6 daltons at 200 ergs/mm^2 that

is calculated on the basis of the 0.05% yield of $\widehat{TT}/T$ at 100 ergs/mm^2 (Table 1, 15). Therefore, it is likely that in L5 cells the discontinuity in daughter strand is due to a gap opposite each dimer that remains unexcised on the parental strand, as Rupp and Howard-Flanders (14) have demonstrated in bacterial recombinational repair. Small discontinuous DNA so produced differs from normal short replicating segments, since the former exhibits a dose-dependent reduction in Mn (unpublished data).

Fig. 2B shows the DNA profiles after an incubation of irradiated cells for 24 hours. This length of time is assumed to be sufficient to allow the cells to complete DNA synthesis, since it proceeds at about 1/3 of the normal rate (unpublished data). In the absence of caffeine, the originally slow sedimenting ^{3}H-DNA was almost converted to the material in size of the ^{14}C-template (Fig. 2B; cross). This implies that gaps in daughter DNA are somehow sealed during or after DNA synthesis. However, 2 mM caffeine, being present during the postirradiation incubation, completely prevents sealing (Fig. 2B; closed triangle), since the profile remains unchanged at the original position where the gapped 2-hour ^{3}H-DNA sediments (Fig. 2A; closed circle). One mM caffeine prevents it less effectively (Fig. 2B; open triangle). Moreover, one can find in Fig. 2 that even 2 mM caffeine does not interrupt with the normal chain growth of replicating DNA up to the interdimer length. By contrast, the identical experiment with HeLa S3

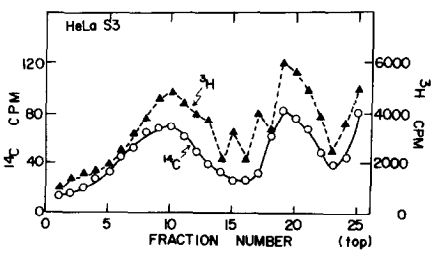

Figure 3. Alkaline sucrose sedimentation profile of the DNA from HeLa S3 cells that were prelabeled with ^{14}C-TdR, synchronized with FUdR, irradiated with 200 ergs/mm^2 at the late G1 window and immediately incubated in the medium containing 25 μCi/ml ^{3}H-TdR and 2 mM caffeine for 24 hours. Note that newly synthesized ^{3}H-DNA (▲) sediments as the ^{14}C-parental DNA (o) and about halves of both DNAs become smaller by degradation during the period (the 17th-23rd fractions).

cells (Fig. 3) does not reveal such a characteristic inhibition of molecular sealing by 2 mM caffeine even after a 24-hour incubation.

DISCUSSION The results presented here indicate that excision-defective mouse L cells bypass the UV photoproduct in their DNA while all the DNA of cells is replicated once. As the result, gaps in the newly replicated strands of DNA are formed and sealed during or after DNA synthesis. This postreplication repair is assumed to resemble bacterial recombinational repair (14). Cleaver and Thomas(5) and Meyn and Humphrey (11) have reported this kind of evidence for the bypass of the photoproduct in Chinese hamster V79 and CHO cells.

Caffeine-sensitive recovery of colony-forming ability of mouse L cells (6, 13, Fig. 1) is correlated with the postreplication repair in terms of the bypass of the photoproduct, with which caffeine interrupts (Fig. 2). It is unlikely from Fig. 2 that caffeine interfers merely with the chain linkage associated with the normal replication in irradiated cells (5), because it proceeds at least up to the interdimer length. Fig. 3 confirms Cleaver's results (4) indicating that caffeine exerts no effect on repair replication in HeLa cells and also suggests that the bypass mechanism may differ substantially from excision repair. Rather, caffeine may interrupt specifically with some step(s) of the complicated post-replication repair by yet unknown mode of its action.

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