REPAIR OF MITOMYCIN C DAMAGE TO DNA IN MAMMALIAN CELLS AND ITS IMPAIRMENT IN FANCONI'S ANEMIA CELLS

Yoshisada Fujiwara and Mariko Tatsumi

Department of Radiation Biophysics, Kobe University
School of Medicine, Ikuta-ku, Kobe 650, Japan

Received July 21,1975

SUMMARY Repair of DNA cross-links by mitomycin C (MMC) was studied in mammalian cells. Skin cells from a patient with Fanconi's anemia (FA9 cells) were about 6 times as sensitive to MMC killing as HeLa S3 cells with normal excision repair ability, while excision-reduced mouse L and human xeroderma pigmentosum (XP2OS) cells were more resistant to it than HeLa S3 cells. Alkaline sucrose sedimentation of DNA revealed that perhaps half-excision of cross-links and its repair occurred efficiently until 4 h of post-MMC time in L-cells and, though more slowly, in HeLa S3 cells. Thus, the excision repair pathway is the first step of the cross-link repair in mammalian cells, but it seems different from the uvrA-dependent pathway in E. coli, since XP2OS cells survived MMC almost normally. Contrarily, FA9 DNA sedimented much faster at 4 h of post-MMC time, suggesting a possible impairment in FA cell's ability to unhook cross-links.

Psoralen-plus-light, MMC, nitrogen- and sulfer-mustards produce interstrand cross-links in DNA of bacteria [2, 3, 11, 13, 20] and animal cells [1, 15]. These cross-links are released from the DNA in wild-type bacteria during post-treatment incubation, but not in uvrA$^-$ or uvrB$^-$ mutants, indicating that dimer-excision enzymes are also involved in the removal of cross-links [2, 11-13, 20]. In this respect, Cole [4] and Howard-Flanders and Lin [9] have recently shown that the repair of psoralen cross-links follows a two-step process that involves the uvrA (or B) gene-dependent excision of the one sides of the cross-links, followed by the recA gene-controlled strand exchanges between homologous duplexes.

Mouse L-cells possess a greatly reduced level of excision repair of UV photoproducts [8, 10, 19]. Reid and Walker [15] have, however, shown that the repair of sulfer mustard cross-links in the same cells appears to involve the rapid excision of the one sides of the corss-links, followed by the slow process leading to the ultimate release of diguaninyl moieties from the DNA. Yet, the whole mechanism of the cross-link repair in mammalian cells is uncertain. Recently, Sasaki

and Tonomura [18] have demonstrated that lymphocytes from patients with FA* show an extraordinarily high susceptibility to chromosome breakage only by cross-linking agents such as MMC, nitrogen mustard and psoralen-plus-light, whereas they show normal susceptibility to monofunctional decarbamoyl MMC, 4-nitroquinoline-1-oxide (4NQO) and UV. This finding possibly suggests that FA cells may have some defect in the repair of interstrand cross-links, inspite of normal incision and resynthesis for UV-induced pyrimidine dimers [14]. Thus, the relevance of the cross-link repair with the dimer-excision repair in mammalian cells is still meager.

This communication will describe the repair of MMC damage to DNA in mammalian cells with different excision-repair capacities and in FA cells by means of survival assay and alkaline sucrose sedimentation of DNA. The results will show that mammalian cells possess an efficient repair pathway for the cross-links which seems different from the dimer-excision repair, and that such repair may be impaired in an FA cell line.

MATERIALS AND METHODS

The cell lines used were mouse L, HeLa S3, xeroderma pigmentosum (SV40-transformed XP2OS, supplied by Dr. H. Takebe, Osaka University, Osaka)[19], and FA9 (supplied by Dr. M. S. Sasaki, Tokyo Medical and Dental University, Tokyo) [18]. The former two were cultured in Ham's F10 medium (GIBCo, Cat. No. F-11) plus 10% calf serum, XP2OS in Eagle's minimal essential medium (GIBCo) plus 15% fetal calf serum, and FA9 in L-15 (Leibovitz) medium (GIBCo, Cat. No. H-13) plus 10% each of fetal and agamma calf sera in Falcon plastic Petri dishes (Div. of BioQuest, Oxnard, Calif.) in a water-saturated atmosphere of 5% CO_2 in air at 37°C [5].

For MMC survival, the appropriate numbers of trypsinized cells in log-growth phase were plated in Falcon dishes and incubated for 4 to 6 h before treatment with MMC (final concentration, 0 to 3 μg/ml) in isotonic phosphate-buffered saline (PBS), pH 7.2, for 1 h at 37°C. After treatment, the cells were washed twice with PBS, and incubated in growth medium for 12 to 28 days until macroscopic colonies developed [5].

Alkaline sucrose sedimentation of cellular DNA was carried out in the following way. Exponentially growing L, HeLa S3, and FA9 cells were prelabeled for 48 h with 0.05 μCi/ml of [2-^{14}C]thymidine (48.4 mCi/mmole, The Radiochemical Centre, Amersham, England). The labeling medium was removed and replaced with normal medium for several hours prior to the 1 h treatment with 0 or 3 μg/ml of MMC. Control and treated cells were lysed immediately, otherwise reincubated for additional 2 and 4 h following twice washes of the plates with PBS, to see further changes in sedimentation characteristics. The procedures for lysing cells and centrifuging DNA through a 5 to 20% alkaline sucrose gradient were described in detail previously [6-8], and briefly in the legend to Fig. 2. The denatured DNA in the final lysate was centrifuged at 35000 rpm for 1 h at 20°C in an SW50.1 head of a Beckman Model L5-50 ultracentrifuge (Beckman

* FA is a rare autosomal recessive hereditary disease characterized by pancytopenia, diverse congenital malformations, a predisposition to leukemia, and spontaneous chromosome breakage.

Instruments, Inc., Palo Alto, Calif.). The radioactivity of acid-insoluble material from sucrose fractions was measured in PPO-POPOP-toluene in a Packard Model 3330 liquid scintillation spectrometer [7].

RESULTS AND DISCUSSION

Fig. 1 shows survival curves after the 1 h treatment with MMC. The present FA9 cells are about 6, 14 and 20 times as sensitive to MMC killing as HeLa S3, XP2OS and L-cells, respectively, under the conditions. Further, L- and XP2OS cells with greatly reduced levels of excision repair of UV damage have greater potentials for tolerating MMC damage than do HeLa S3 cells, suggesting that the first UV endonuclease step of excision repair of the dimers may not be involved in the

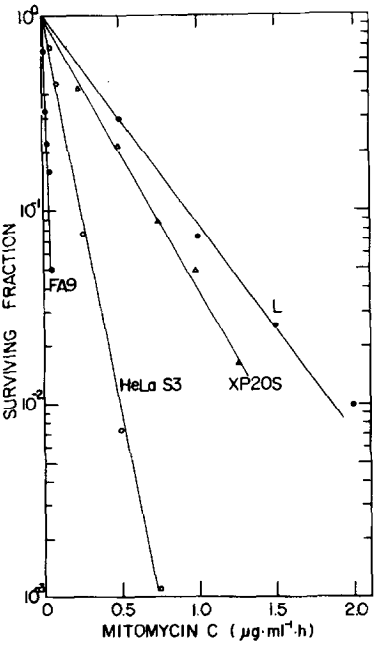

Figure 1. MMC survival curves. Exponentially growing mouse L, human XP2OS, HeLa S3, and FA9 cells were typsinized, plated in Falcon Petri dishes (60 x 15 mm), and treated with graded concentrations of MMC in PBS for 1 h at 37°C. After washed twice with PBS, the plates were further incubated for 12 to 28 days until visible colonies developed. Each point is the mean of 3 to 5 experimental determinations. Plating efficiencies and mean lethal MMC concentrations (which reduce survivals to 1/e) are: L (⊙—⊙), 88-100%, 0.40 μg/ml·h; XP2OS (Δ—Δ), 5.1-9.8%, 0.30 μg/ml·h; HeLa S3 (○—○), 74-93%, 0.12 μg/ml·h; FA9 (●—●), 0.8-1.8%, 0.02 μg/ml·h.

repair of MMC damage, probably interstrand cross-links in L- and XP2OS (perhaps in HeLa S3) cells. This pathway may be different from the uvrA-dependent half-excision of DNA cross-links in E. coli (see "Introduction"). Moreover, it is highly suggestive that an extremely MMC-sensitive FA9 cells must be somehow defective in such a characteristic cross-link repair mechanism.

Reid and Walker [15] have reported that "one-arm unhooking" of sulfer mustard cross-links occurs very rapidly in L-cells. To see whether L-cell DNA is degraded by excision of one sides of cross-links (and monoadducts), [^{14}C]thymidine-prelabeled cells were treated with 3 μg/ml of MMC and incubated in normal medium up to 4 h. In Fig. 2A, treated L DNA sedimented more slowly immediately after MMC than control, and its profile returned almost to the control by 4 h. In L-cells, therefore, single DNA chains are presumed to be cleaved endonucleolytically during repair of MMC damage which proceeds rather quickly. This efficient repair may account for the highest MMC survival of L-cells in Fig. 1, and the repair time (4 h) may be reconciled with the "one-arm unhooking" time (half-life, ~2 h) of sulfer mustard cross-links in the same cells [15]. Almost the similar, but slower change in the sedimentation rate also occurs in MMC-treated HeLa S3 cells (Fig. 2B).

In particular contrast, MMC-treated FA9 DNA sedimented much faster at 4 h of post-MMC incubation (Fig. 2C): the DNA profile consists of two discrete peaks at around 9.3×10^8 and 2.8×10^8 daltons. The highest-molecular-weight DNA that is dissociated from unlinked molecules at 4 h in FA9 cells presumes that the cells cannot, perhaps, carry out the half-excision of MMC cross-links.

In E. coli, the repair of psoralen cross-links in their DNA requires the excision-recombination sequence: the first uvrA (or B)-dependent half-excision and the subsequent recA gene-controlled DNA exchanges between homologous duplexes [4, 9]. In the present mammalian cell experiments, however, excision-reduced L-cells degrade MMC-treated DNA and repair it more efficiently (Fig. 2A) than, and they survive MMC more efficiently (Fig. 1) than HeLa S3 cells. Furthermore, uvr$^-$-mimic XP2OS cells [19] survive MMC to a considerable extent (Fig. 1). These results together suggest that the half-excision of cross-links in mammalian cells may be carried

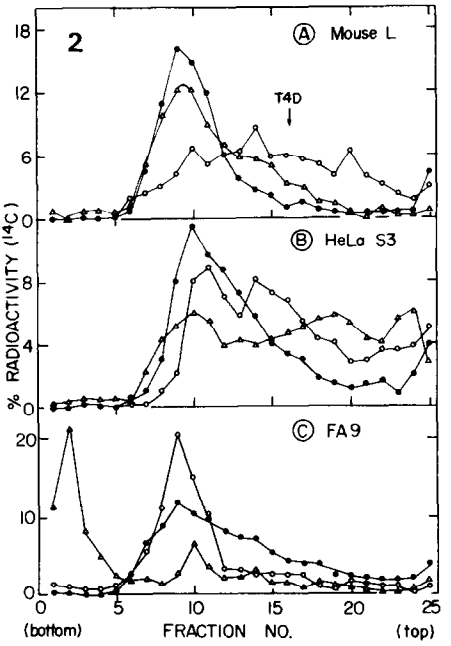

Figure 2. Alkaline sucrose sedimentation profiles of the DNA's from MMC-treated or untreated cells. Exponentially growing mouse L (A), HeLa S3 (B), and FA9 cells (C) were prelabeled with 0.05 μCi/ml of [^{14}C]thymidine, treated with 0 or 3 μg/ml of MMC in PBS for 1 h at 37°C. The cells were lysed in 0.25% sodium dodecylsulfate, 0.01 M EDTA and 0.15 M sodium bicarbonate, pH 8.0, and digested with 2 mg/ml of preheated Pronase (Kaken Chemical Co., Tokyo) for 3 to 4 h at 37°C (6-8). After addition of 0.1 ml of 3 M NaOH per 1 ml of the lysate (adjusted to contain ~5,000 lysed cells/0.2 ml : <0.1 μg denatured DNA), a 0.2-ml aliquot was layered on top of 4.8 ml of 5 to 20% alkali-sucrose gradient containing 0.8 M NaCl, 0.2 M NaOH, 0.01 M EDTA, and 0.015 M sodium p-aminosalicylate, pH 12.5 (6-8). The loaded polyallomer tubes were centrifuged at 35,000 rpm for 1 h at 20°C in an SW50.1 rotor of a Beckman Model L5-50 ultracentrifuge using labeled T4D DNA for internal reference. After the run, 10-drop fractions were collected from the bottoms of the tubes, followed by measurement of radioactivity. ●—●, untreated, lysed immediately; o—o, treated, lysed immediately; Δ—Δ, treated, lysed at 4 h of post-MMC incubation.

---

out by a unique system independent of the UV endonuclease for pyrimidine dimers.

Fig. 2C shows probably that the present FA9 cells are unable to cut the cross-linked DNA during 4 h of post-MMC incubation. This finding seems compatible with Sasaki's data [17] indicating that MMC-induced pre-chromosomal aberration lesions, probably cross-links, in FA cells are not lost during the recovery time over the whole G1 period, whereas decarbamoyl MMC-

induced lesions (only monoadducts) are lost. Such a pattern of chromosome response in FA cells to MMC is very much like that in XP (unable to excise 4NQO lesion [19]) to 4NQO [16], although nature of damage to DNA is different.

Accordingly, the present FA9 cells are assumed to have an impairment in unhooking the one side of the cross-link, and this impairment might be the major cause of FA's high sensitivity to killing (Fig. 1) and chromosome aberration formation [18] by MMC. The precise determination of the defective repair site in FA cells will be the subject to future study.

ACKNOWLEDGEMENTS

We are very grateful to Dr. M. S. Sasaki, Tokyo Medical and Dental University, Tokyo, for his generous supply of FA9 culture cells and his valuable comments and suggestions to this work, and to Dr. S. Kondo, Osaka University School of Medicine, Osaka, for his criticism and discussions. A partial support of this work by a Grant-in-Aid for Cancer Research from the Ministry of Education, Japan, is also acknowledged.

REFERENCES

1. Ben-Hur, E., and Elkind, M. M. (1973) Biochim. Biophys. Acta, 331, 181-193.
2. Boyce, C. P., and Howard-Flanders, P. (1964) Z. Verbungsl., 95, 345-350.
3. Cole, R. S. (1971) Biochim. Biophys. Acta, 254, 30-39.
4. Cole, R. S. (1973) Proc. Natl. Acad. Sci. USA, 70, 1064-1068.
5. Fujiwara, Y. (1967) J. Cell Physiol., 70, 291-300.
6. Fujiwara, Y. (1972) Exptl. Cell Res., 75, 483-488.
7. Fujiwara, Y. (1975) Biophys. J., 15, 403-415.
8. Fujiwara, Y., and Kondo, T. (1972) Biochem. Biophys. Res. Commun., 47, 557-564.
9. Howard-Flanders, P., and Lin, P.-H. (1973) Genetics, 73 (suppl.), 85-90.
10. Klimek, M., (1966) Photchem. Photobiol., 5, 603-611.
11. Kohn, K. W., Steigbigel, N. H., and Spears, C. L. (1965) Proc. Natl. Acad. Sci. USA, 53, 1154-1161.
12. Kondo, S., Ichikawa, H., Iwo, K., and Kato, T. (1970) Genetics, 66, 187-217.
13. Lawley, P. D., and Brooks, P. (1965) Nature, 206, 480-483.
14. Poon, P. K., O'Brien, R. L., and Parker, J. W. (1974) Nature, 250, 223-225.
15. Reid, B. D., and Walker, I. G. (1969) Biochim. Biophys. Acta, 179, 179-188.

16. Sasaki, M. S. (1973) Mutation Res., 20, 291-293.
17. Sasaki, M. S. (1975) Manuscript submitted for publication.
18. Sasaki, M. S., and Tonomura, A. (1973) Cancer Res., 33, 1829-1836.
19. Takebe, H., Nii, S., Iida-Ishii, M., and Utsumi, H. (1974) Mutation Res., 25, 383-390.
20. Venitt, S. (1968) Biochem. Biophys. Res. Commun., 31, 355-360.