Photodynamic effects of haematoporphyrin derivative on DNA repair in murine L929 fibroblasts

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Illumination with red light of murine L929 fibroblasts that had been sensitized with haematoporphyrin derivative caused DNA single-strand breaks after a lag time of about 20 min, as revealed by alkaline elution. The cells appeared not to be capable of recovering from this damage. The photodynamic effect of haematoporphyrin derivative on DNA repair was assessed by monitoring the repair kinetics of DNA damage induced by either X-rays, u.v. light (254 nm) or methyl methanesulphonate treatment subsequent to a non-DNA-damaging photodynamic treatment with haematoporphyrin derivative. On 'post-incubation', the normally rapid repair of X-ray-induced DNA strand breaks did not occur, whereas with u.v. light and methyl methanesulphonate treatment after photodynamic treatment prolonged post-incubation resulted in an increase in the number of strand breaks rather than the normally observed decrease. This clearly shows that, after a photodynamic treatment with haematoporphyrin derivative that itself did not cause strand breaks, excision repair in L929 cells is severely inhibited at a stage beyond the incision step.

INTRODUCTION

Photodynamic therapy with haematoporphyrin derivative (HPD) as sensitizer causes destruction of malignant tumours. Direct tumour-cell inactivation and microvascular disruption, possibly caused by damage to endothelial cells, have both been shown to contribute to this effect [1–3]. A detailed study of the photodynamic effects of HPD on various cellular parameters is therefore essential in order to elucidate the ultimate cause of cell inactivation in photodynamic therapy. It has been shown that the active membrane transport systems for 2-aminoisobutyric acid and cycloleucine, the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase and mitochondrial sites such as the proton-translocating ATPase are among the most sensitive targets [4–6]. In addition, damage to DNA, namely single-strand breaks, DNA–protein cross-links and induction of sister chromatid exchanges, has also been described [7–11]. In CHO and NHIK 3025 cells, single-strand breaks can be repaired [7–9]. Gomer [9] examined the repair kinetics of photodynamically induced strand breaks in CHO cells at different doses. At the highest dosage of HPD photoradiation used, repair no longer took place, and he suggested that repair enzymes might have been inactivated. In cytosols prepared from normal and tumour tissue [12] and with purified enzymes [13] it has indeed been shown that enzymes like DNA polymerases, which are assumed to be involved in repair, can be photodynamically inhibited. In intact cells, however, a possible inhibition of DNA repair systems has never been demonstrated unambiguously.

This prompted us to determine, in L929 cells, the photodynamic effect of HPD on the repair kinetics of DNA damage, induced by X-rays, u.v. light and the methylating agent MMS.

MATERIALS AND METHODS

N.C.T.C. murine fibroblasts (clone L929, A.T.C.C. number CCL1), tissue-culture products and newborn-calf serum were obtained from Flow Laboratories. HPD was purchased from Photofrin Medical (Raritan, NJ 08869, U.S.A.), [^{14}C]thymidine (52 mCi/mmol) from Amersham, Pico-Fluor 30 from Packard Instruments, MMS from Merck, and filters (Metricel, 0.45 μm pore size) from Gelman. All other chemicals were from Boehringer, Sigma or Merck and were of analytical grade.

L929 cells were grown as described previously [4] in the presence of 0.1 μCi of [^{14}C]thymidine in 4 ml of medium. After 72 h the medium was removed and the cells were cultured for another 24 h without radioactive thymidine.

Confluent cell layers (about 2×10^6 cells) were incubated with 10 μg of HPD/ml and illuminated as described previously [4]. U.v.-light treatment was performed with a Philips TUV lamp, predominantly emitting at 254 nm. The dose rate was 0.22 J/s per m^2 and the total dose was 15 J/ m^2 . In control experiments it appeared that illumination with this light source did not cause photoexcitation of HPD. MMS treatment was performed by incubation of the cells with 2 mM-MMS in DPBS for 30 min at 37 °C in a CO_2 incubator. X-irradiation of the cells, covered with ice-cold DPBS, was done with a Muller RT-100 X-ray apparatus equipped with a 0.3 mm Al filter. The dose rate was 15 rad/s, and the total dose at the cell layer was 500 rad.

Either directly after the treatments or after post-incubation in culture medium at 37 °C in the CO_2 incubator, the cell layer was washed twice with ice-cold DPBS. Subsequently DNA strand breaks were assayed by the alkaline-elution technique described by Kohn

Abbreviations used: HPD, haematoporphyrin derivative; DPBS, Dulbecco's phosphate-buffered saline; MMS, methyl methanesulphonate.

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et al. [14]. Briefly, after lysis of 10^6 cells on the filters with 0.2% Sarkosyl, the residue remaining on the filters was treated with proteinase K. DNA was eluted with 0.1 M-tetrapropylammonium hydroxide buffer, pH 12.2, at a flow rate of 0.66 ml/h. Before counting the radioactivity of the filters, they were treated as described by Kohn *et al.* [15] in order to minimize chemiluminescence. The radioactivity of the filters and the eluted fractions was counted in Pico-Fluor 30 scintillant containing 0.7% acetic acid, utilizing an Isocap 300 liquid-scintillation counter. Strand-break frequency was calculated as described by Zwelling *et al.* [16], by comparing the elution rate of DNA from HPD-treated cells with that from cells that had received 300 rad of X-rays.

RESULTS

The induction of DNA strand breaks in L929 fibroblasts by photodynamic treatment with $10 \mu\text{g}$ of HPD/ml is shown in Fig. 1. After a lag time of about 20 min a progressive increase in elution velocity could be observed. After 1 h of illumination the strand-break frequency was about 1.5 breaks/100 GDa of DNA. The elution velocity of DNA from cells illuminated for 15 min did not change after post-incubation for 0, 1, 2, 4, 10, 16 or 24 h. Post-incubation after a DNA-damaging photodynamic treatment (illumination period 30 min or longer), on the other hand, always resulted in a further increase of the number of strand breaks (Fig. 1). In control experiments it appeared that cells illuminated for 2 h without prior incubation with HPD or cells incubated with HPD but kept in the dark gave the same elution rate as non-treated cells.

In order to determine the photodynamic effect of HPD on DNA repair, DNA damage was induced by X-rays, u.v. light or MMS treatment after a prior, brief (15 min

Fig. 1. Photodynamic effect of HPD on the alkaline-elution kinetics of DNA from L929 cells

○, Dark control; △, 20 min illumination, with or without post-incubation; □, 30 min illumination, no post-incubation; ▽, 60 min illumination, no post-incubation; ▼, 60 min illumination, 4 h post-incubation.

illumination) photodynamic treatment that by itself did not cause strand breaks. The repair kinetics of strand breaks induced by X-rays alone are shown in Fig. 2(a). Immediately after treatment a considerable number of breaks was present. After 3 h of post-incubation the strand breaks had completely disappeared. The effect of photodynamic treatment prior to X-irradiation is shown

Fig. 2. Effect of illumination of HPD-preincubated L929 cells on the repair kinetics of X-ray-induced single-strand breaks

(a) 500 rad of X-ray irradiation alone; ●, no post-incubation; □, 1 h post-incubation; ■, 3 h post-incubation. (b) Photodynamic treatment (15 min illumination) followed by 500 rad of X-ray irradiation; ●, no post-incubation; ■, 3 h post-incubation; ▽, cells incubated with $10 \mu\text{g}$ of HPD/ml but not illuminated, followed by 500 rad of X-rays and 3 h post-incubation.

Fig. 3. Photodynamic effect of HPD on the repair kinetics of u.v.-light-induced DNA damage in L929 cells

(a) U.v. irradiation (15 J/m^2) only. Post-incubation period: $\circ$, none; $\triangle$, 5 min; $\square$, 30 min; $\blacktriangle$, 3 h; $\bullet$, 16 h; $\blacksquare$, 24 h. (b) Photodynamic treatment (15 min illumination) followed by 15 J of u.v. irradiation/ m^2 . Post-incubation period: $\circ$, none; $\square$, 30 min; $\blacksquare$, 24 h. ∇ , Cells incubated with $10 \mu\text{g}$ of HPD/ml but not illuminated, followed by 15 J of u.v. light/ m^2 and 24 h of post-incubation.

in Fig. 2(b). Immediately after treatment the elution rate was the same as after treatment with X-rays alone. Post-incubation of 3 h did not change this elution pattern, indicating that X-ray-induced strand breaks were no longer repaired. Control experiments exhibited that incubation of the cells with HPD without illumination had no influence on the repair kinetics of X-ray-induced breaks (Fig. 2b).

The effect of the photodynamic action of HPD on repair of u.v.-light-induced lesions is shown in Fig. 3. Immediately after u.v. irradiation alone, only a very limited number of strand breaks could be detected (Fig. 3a). During post-incubation the number of strand breaks initially increased. Thereafter it decreased again, and,

after 24 h of post-incubation, strand breaks were no longer detectable. When u.v. irradiation was preceded by a 15 min photodynamic treatment, the number of strand breaks also increased during the first 30 min of post-incubation. Longer post-incubations, however, resulted in a further increase of the strand-break frequency instead of the decrease observed after u.v. light alone (Fig. 3b). Incubation with HPD without illumination had no effect upon the repair kinetics of u.v.-light-induced DNA damage in L929 cells.

The photodynamic effect on the repair of MMS-induced DNA damage was similar to its effect on u.v.-light-induced damage. With MMS alone the number of strand breaks first increased during post-incubation

Fig. 4. Photodynamic effect of HPD on the repair kinetics of MMS-induced DNA damage in L929 cells

(a) MMS treatment (2 mM) only. Post-incubation period: $\circ$, none; $\square$, 1 h; $\bullet$, 2 h; $\blacksquare$, 4 h. (b) MMS treatment subsequent to photodynamic treatment. Post-incubation period: $\circ$, none; $\square$, 1 h; $\blacksquare$, 4 h. ∇ , Cells incubated with $10 \mu\text{g}$ of HPD/ml but not illuminated, followed by exposure to MMS and 4 h of post-incubation.

and subsequently decreased. After 4 h of post-incubation repair was complete (Fig. 4a). With photodynamic treatment before exposure to MMS the strand-break frequency increased during the entire post-incubation period (Fig. 4b).

DISCUSSION

Photodynamically induced DNA damage in the form of alkali-labile sites, strand breaks, DNA-protein cross-links and induction of sister chromatid exchanges has been reported previously [7-9, 11]. At physiological pH, guanine is by far the most photosensitive base [10] and therefore it may be assumed that the DNA damage results mainly from guanine degradation. As shown in Fig. 1, in L929 cells strand breaks are formed by the photodynamic action of HPD. A convex curvature of the elution curve suggests the presence of alkali-labile sites converted during elution to single-strand breaks [15]. Therefore the results shown in Fig. 1 suggest that at least part of the photodynamically induced DNA damage should be interpreted as alkali-labile sites, presumably caused by photo-oxidative base (guanine) damage.

CHO and NHIK 3025 cells, at least at low photodynamic doses, can recover from photodynamically induced DNA strand breaks by post-incubation after illumination [7,9]. However, in the present study utilizing L929 cells, repair of photodynamically induced DNA strand breaks never occurred. On the contrary, during post-incubation the number of strand breaks always increased, even after the smallest illumination period that caused detectable DNA damage (about 20 min). A possible explanation is that repair in these cells is very sensitive to the photodynamic action of HPD. To investigate this possibility, the photodynamic effects on the well-documented repair systems, operative after DNA-damaging treatment with X-rays, u.v. light and the alkylating agent MMS were studied. Because DNA damage induced by each of these three different entities has a different repair pathway (for reviews see [18,19]), the photodynamic effect of HPD was studied with all three treatments separately. For these combinations an HPD+light treatment is chosen that itself did not cause detectable DNA damage, because such damage would have obscured the detection of damage and subsequent repair induced by the second treatment. Therefore an illumination time of 15 min was used (Fig. 1). Post-incubation up to 24 h after this phototreatment did not result in detectable DNA damage.

X-rays induce directly single-strand breaks [19] that are subsequently repaired by enzymes or enzyme systems with polymerase and ligase activities [20,21]. L929 cells appear to recover completely from this damage in about 3 h. When X-rays are preceded by the non-DNA-damaging photodynamic treatment, single-strand breaks are no longer repaired. This indicates an efficient inhibition of one or more of the enzymes involved in this repair.

During treatment with u.v. light, pyrimidine dimers are formed. Repair starts by incision at the sites of the dimers. This incision predominates initially during post-incubation, resulting in an increasing number of strand breaks. Subsequently strand-break frequency decreases again, due to successive exonuclease, polymerase and ligase activities, and, after 24 h, all breaks have disappeared. From the repair kinetic studies after

combination of HPD+light with u.v. light it is apparent that the repair process is strongly inhibited at a point beyond the incision step. This leads to a further increase in the number of breaks, similar to the increase observed in the presence of inhibitors of repair synthesis [22].

MMS causes methylation of the bases guanine and adenine [23]. This modification itself does not give rise to strand breaks. Repair of these lesions includes formation of apurinic sites by the activity of glycosylases and incision by AP-endonucleases (for reviews see [17,18]). Therefore the increased rate of alkaline elution reflects both the presence of alkali-labile sites and of breaks caused by the incision step. This results in the slight convex curvature of the elution curves. The results of post-incubation after treatment with MMS alone or combination with photodynamic treatment are similar to the results observed with u.v. light and its combination with HPD+light. The only difference is that, under our experimental conditions, repair is complete after 4 h when cells were treated with MMS alone. When exposure to MMS was preceded by photodynamic treatment, the incision step was again unaffected, whereas one or more of the successive steps were inhibited.

The above-described inhibitory effects are very similar to the effects of inhibitors of repair such as hydroxyurea and arabinosylcytosine [22] and indicate an efficient inhibition somewhere in the steps following the incision. More detailed experiments are necessary to determine whether repair is completely inhibited in these cells and which of the enzymes involved in these steps are inactivated.

The L929 cells used in the present studies differ significantly from CHO cells with respect to the relative sensitivity of their DNA repair enzymes (as compared with the DNA molecules themselves) to photodynamic damage. Apparently repair in L929 cells is effectively inhibited even before DNA strand breaks can be observed. With CHO cells, on the other hand, quite different results were obtained. After an HPD+light treatment of these cells leading to a strand-break frequency as high as seven breaks/100 GDa of DNA, repair still occurred [8]. This indicates that the relative sensitivity of the repair enzymes in these cells to photodynamic damage is much lower than in L929 cells.

Inhibition of DNA repair is an early event in the time course of development of photodynamic damage in intact L929 cells after incubation with HPD. Inhibition was observed after an illumination period of 15 min. Comparison with previous studies reveals that, at the same dose, only the aminoisobutyric acid- and active Rb⁺-transport systems are also severely impaired [4]. All other studied parameters, namely activities of cytosolic, mitochondrial and lysosomal enzymes, and sizes of ATP and GSH pools, are far less sensitive [24].

Our results suggest that, at least in certain cases, photodynamic therapy in combination with X-rays might be advantageous, as a potentiating effect between these two modalities of therapy can be anticipated, when the DNA repair system of the cells is highly sensitive to photodynamic inactivation. Indeed, Kostron *et al.* [25], working with a rat glioma model, observed a synergistic effect of photodynamic treatment and ⁶⁰Co irradiation. Interestingly, in similar experiments with CHO cells (in which the DNA-repair enzymes are relatively insensitive to photodynamic inactivation, as discussed above)

Bellnier & Dougherty [26] did not observe a synergistic effect of photodynamic treatment and γ -radiation. Rather, their experimental data indicated an independent interaction between the damage induced by ionizing radiation and that induced by HPD photosensitization [26].

This work was supported by a grant of the Netherlands Cancer Foundation (IKW 84-45).

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Received 15 January 1987/27 February 1987; accepted 6 March 1987