DNA REPLICATION AND REPAIR IN TILAPIA CELLS
I. THE EFFECT OF ULTRAVIOLET RADIATION

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SUMMARY

The effect of ultraviolet radiation on a cell line established from the warm water fish Tilapia has been assessed by measuring the rate of DNA synthesis, excision repair, post-replication repair and cell survival. The cells tolerate ultraviolet radiation better than mammalian cells with respect to DNA synthesis, post-replication repair and cell survival. They are also efficient in excision repair, which in other fish cell lines has been found to be at a low level or absent. Their response to the inhibitors hydroxyurea and 1-β-D-arabinofuranosylcytosine is less sensitive than that of other cell lines, yet the cells seem to have very small pools of DNA precursor.

INTRODUCTION

Cell lines derived from fish have attracted considerable interest in recent years. Regan, Carrier, Samet & Olla (1972) studied the cells from two closely related marine fish with very different life spans, which, they found, corresponded to their photoreactivation activities. Mitani, Etoh & Egami (1982) found that cells from goldfish were more resistant to γ-irradiation than mammalian cells; this was consistent with earlier studies on the individual goldfish (Hyodo-Taguchi & Egami, 1969; Purdom & Woodhead, 1973). However, the same cells were found to be more sensitive to ultraviolet (u.v.) radiation than mammalian cells (Mano, Mitani, Etoh & Egami, 1980), although the amount of DNA damage was the same as that found in mammalian cells (Mitani & Egami, 1982; Mano, Kator & Egami, 1982).

Photoreactivation of pyrimidine dimers on DNA has long been a feature of fish cell repair. The work of Hart, Setlow & Woodhead (1977) related the induction of pyrimidine dimers to tumour formation and their photoreactivation to the absence of tumorigenicity. More recent investigations into the lethal and mutagenic effects of radiation and chemicals (Mitani, 1983) have all demonstrated that photoreactivation plays a vital role in survival and transformation of fish cells from the cell lines studied; it was concluded that excision repair was minimal and probably of minor importance (Mano et al. 1980, 1982; Regan et al. 1972).

In this paper we present the results of studies of the effects of u.v. radiation on cells derived from the warm water fish Tilapia. We have investigated the effects of radiation on excision repair, DNA replication, post-replication repair and cell proliferation.

Key words: Tilapia, DNA replication, DNA repair.
MATERIALS AND METHODS

Cell culture

*Tilapia* kidney (TK) cells were originally derived from the hybrid species of *Tilapia mossambica* and *T. nilotica* (Chen et al. 1983). They were routinely cultured in 25-ml plastic flasks in Lebovitz-15 medium supplemented with 10% (v/v) foetal calf serum and antibiotics at 31 °C. For most experiments, cells were transferred to 24-well plate at a 1:5 split ratio, from confluent culture.

Chinese hamster ovary (CHO) cells were cultured in McCoy's medium supplemented with 10% foetal calf serum.

Cell killing

Cells were trypsinized, washed twice and resuspended in cold phosphate-buffered saline (PBS). U.v. radiation was carried out in a 30 mm Petri dish in 1 ml of cold PBS at a dose rate of 1·0 Jm⁻²s⁻¹ for various lengths of time. When a 24-well plate was used, because of the shielding effect of the walls of the well, the dose rate was reduced to 0·9 Jm⁻²s⁻¹. After irradiation the cells were centrifuged and transferred to normal growth medium in either 25-ml flasks or 24-well plates, the number of cells inoculated was recorded. To prevent photoreactivation the cells were kept in the dark during incubation. For proliferation studies the cells in the flasks were trypsinized and counted on subsequent days. The percentage of cells engaged in DNA synthesis was also measured. The cells in the 24-well plates were pulse-labelled with [³H]thymidine (1 μCi/ml, 31 Ci/mmol) for 1 h, then lysed in 0·5 ml of alkaline solution (5% (w/v) sucrose, 0·3 M-NaOH, 0·5 M-NaCl) for 5 min and DNA was precipitated by adding a drop of 50% cold trichloroacetic acid and standing at 4°C overnight. The precipitate was collected by filtering through Whatman 2·4 cm GF/C glass-fibre filters, washed with 5% cold trichloroacetic acid and 95% (v/v) ethanol, dried and counted in a scintillation counter.

Nucleoid sedimentation

The sedimentation behaviour of nucleoids derived from TK cells was studied using the method of Cook & Brazell (1975). Cells were labelled with [³H]thymidine (0·5 μCi/ml, 31 Ci/mmol) for 48 h at 31°C. They were trypsinized, washed twice and suspended in PBS; 50 μl of cell suspension containing 5 x 10⁴ cells was gently layered on 150 μl of a lysis buffer (2 M-NaCl, 0·01 M-EDTA, 0·1% Triton X-100, pH 8·0) on top of a linear sucrose gradient (15% to 30% sucrose, 2·0 M-NaCl, 0·01 M-EDTA, pH 8·0; total volume 4·8 ml). For the titration of DNA supercoils, ethidium bromide (0—10 μg/ml) was present in the lysis buffer as well as in the gradient. The gradients were transferred to a RPS 50-2 rotor and centrifuged at 30000 rev./min on a Hitachi SCP 85H ultracentrifuge at 20°C for 40 min. After centrifugation, 20 fractions were collected directly into counting vials mixed with 5 ml scintillation fluid (66·67% toluene, 33·33% Triton X-100, 5 g PPO, 0·1 g POPOP in 1 litre) and counted on a LKB 1217 Rackbeta liquid scintillation counter.

Rate of DNA synthesis

The method was based on the work of Painter (1977). Cells were generally labelled with [¹⁴C]-thymidine (0·02 μCi/ml, 56 mCi/mmol) for 48 h. The radioactive medium was replaced with fresh medium and the cells were further incubated for 4 h. After u.v. radiation at various doses, cells were pulse-labelled with [³H]thymidine (20 μCi/ml, 31 Ci/mmol) for 10 min at various times (0, 30 min, 1 h, 2 h), they were then lysed with alkali, precipitated with trichloroacetic acid, filtered and counted.

Alkaline sucrose gradient sedimentation

Alkaline sucrose gradient sedimentation was used in assessing the presence of single-strand breaks in the parental DNA and post-replication (daughter strand) repair. The gradient contained 0·3 M-NaOH, 0·5 M-NaCl, 0·01 M-EDTA, 5% to 20% sucrose (pH 12·3) and was always kept at 4°C. After centrifugation, 20 fractions were collected. DNA was precipitated by adding a drop of 50% ice-cold trichloroacetic acid. The fractions were kept in the cold overnight, then filtered through
Whatman 2-4 cm GF/C glass-fibre filters. They were washed with 5% cold trichloroacetic acid and 95% ethanol and counted in a liquid scintillation counter phage λ DNA was used as a molecular weight marker.

Assay of DNA single-strand breaks

The method was based on those of Collins, Schor & Johnson (1977) and Johnson & Collins (1978). The cells were generally labelled with $[^3]H$thymidine (0-5 μCi/ml, 31 Ci/mmol) for 48 h before u.v. radiation. The cells were incubated with or without inhibitors for 1 h before radiation. After u.v. radiation followed by incubation with or without inhibitors, the cells were scraped off the surface, washed with PBS and suspended in 0.2 ml PBS. The sample were layered on top of an alkaline sucrose gradient and centrifuged immediately at 30,000 rev./min for 30 min at 4°C.

Post-replication repair

The DNA was uniformly labelled with $[^4]C$thymidine (0.02 μCi/ml, 56 mCi/mmol) for 48 h. After u.v. radiation, the cells were pulse-labelled with $[^3]H$thymidine (20 μCi/ml, 31 Ci/mmol) for 30 min. The medium was replaced by fresh medium containing 10 mM-thymidine and chased from 0 to 1 h. After the chase period, the cells were scraped off the surface and lysed on top of an alkaline sucrose gradient for 12 h in the cold. The gradients were then centrifuged for 3 h at 30,000 rev./min at 4°C.

RESULTS

The effect of u.v. radiation on cell proliferation

TK cells do not form well-defined colonies. It is therefore not possible to construct a dose-response curve for single cell survival. Instead, by counting cell number each day after irradiation, the effect of u.v. on proliferation can be established (Fig. 1). We found that on the first day after u.v. radiation, there was a dose-dependent decrease in the number of attached cells in all the flasks except the control. TK cells tolerate 4.5 Jm$^{-2}$ of u.v. well enough to resume the control proliferation rate after 2 days. Cell irradiated with 9 Jm$^{-2}$ recovered to the starting cell number after 3 days, while cultures exposed to higher u.v. doses showed no recovery. This was further confirmed by $[^3]H$thymidine pulse-labelling the cultures 1 day and 2 days post-irradiation to assess replicative ability (Fig. 2).

Inhibition of replicative DNA synthesis by u.v. light and the effect metabolic inhibitors

The inhibition of semi-conservative DNA synthesis by u.v. radiation has been well documented in both bacteria (Swenson & Setlow, 1966) and mammalian cells (Cleaver, Kaufmann, Kapp & Park, 1983). In general, DNA synthesis decreased immediately after u.v. radiation. Fig. 3 shows how the rate of DNA synthesis during a 10-min pulse label was affected by u.v. This was the method used by Painter (1977) to assess the damaging effect of mutagens on DNA. Our results on CHO cells were similar to those reported by Meyn, Hewitt, Thomson & Humphrey (1976) and Doniger (1978). On the other hand, TK cells showed a drop in their rate of DNA synthesis immediately after 9 Jm$^{-2}$ irradiation (see 0 h in Fig. 3), reached the minimum at 30 min, but recovered within 2 h, which is comparable to CHO after 4.5 Jm$^{-2}$, but considerably faster than CHO after the same dose of u.v. The accumulation of $[^3]H$thymidine counts during the first 30 min post-irradiation is shown
Fig. 1. The proliferation of TK cells after u.v. radiation. The cells were trypsinized and suspended in PBS, then irradiated with 0(Δ—△), 4·5(●—○), 9(□—□), 18(●—●), 36(△—△) and 45 Jm⁻²(■—■) of u.v. They were transferred into growth medium in several flasks and the cell numbers were recorded. After 1, 2 and 3 days, one flask of each dose was trypsinized and the cell numbers were counted. The cell number plated in the control flask was taken as 100%.

in Fig. 4. In both types of cells u.v. induced a dose-related decline in the incorporation of [³H]thymidine, TK cells being slightly more sensitive (Fig. 4A, B).

The metabolic inhibitor hydroxyurea (HU) inhibits DNA synthesis by blocking the enzyme ribonucleotide reductase (Sjoeberg, Reichard, Graeslund & Ehrenberg,

Fig. 2. The incorporation of [³H]thymidine into TK cells 1 (Δ—△) and 2 (●—○) days after u.v. radiation. The cells after irradiation in PBS were plated in a 24-well plate. One and two days after irradiation the cells were pulse-labelled for 30 min with [³H]thymidine. The number of counts registered on unirradiated control is taken as 1. The error bar indicates the standard deviation of four experiments.

Fig. 3. The rate of DNA synthesis for TK and CHO cells. The cells were generally labelled with [¹⁴C]thymidine, then replated into 24-well plate in subconfluence for 12 h without [¹⁴C]. u.v. radiation was carried out in PBS followed by a 10-min pulse label with [³H]thymidine in complete medium at 0, 30 min, 1 h and 2 h after u.v. The ratio of [³H]/[¹⁴C] for control cells was taken as 100%. The standard deviation of four experiments was ±10% for TK (——) cells and ±13% for CHO (-----) cells. u.v. dose: 4·5(▲), 9(△), 18(■), 36(◇) and 45 Jm⁻²(●).
It is widely used in cell synchronization (Adams & Lindsay, 1967; Skoog & Nordenskjoeld, 1974) and may, with caution, be used to measure unscheduled DNA synthesis (Smith & Hanawalt, 1976). Our results show that in CHO cells (Fig. 4A), as in many other cell lines, hydroxyurea inhibited over 90% of semi-conservative DNA replication (Timson, 1975). In TK cells (Fig. 4B), however, without u.v. hydroxyurea (10 mM) suppressed only 80% of total DNA synthesis. The inhibition did not increase with increasing dose of u.v.

1-β-D-Arabinofuranosylcytosine (araC) as araCTP inhibits DNA synthesis either by affecting the DNA polymerase or as a chain terminator (Chu & Fisher, 1962; Waquar, Burgoyne & Atkinson, 1971; Monparler, Rossi & Labatan, 1973; Skoog & Nordenskjoeld, 1974); it suppressed DNA replication activities by 95% in CHO (Fig. 4A), but in TK cells (Fig. 4B) the effect of araC is not quite as pronounced. However, treatment with HU and araC combined resulted in more than 90% inhibition of incorporation in both cell types.

We also tried to determine the inhibitory effects of HU and araC at various concentrations on CHO and TK cells. The results showed that HU (Fig. 5A) was more effective with CHO than TK cells at concentrations from $10^{-1}$ to $10^{-4}$ M, the difference was approximately 15% throughout. araC (Fig. 5B) at $10^{-2}$ M inhibited DNA synthesis in both cell lines by more than 90%; however, the inhibitory effect was
diluted away almost completely at $10^{-6}$ M, while at this concentration there was still a suppression of 60% of DNA synthesis in CHO cells. The inhibition of DNA synthesis by HU and araC is reversible (Collins & Johnson, 1981; Downes & Collins, 1982). The effectiveness of the drugs probably depends on several factors, including the sizes of internal deoxyribonucleotide pools (Skoog & Nordenskjoeld, 1974), and the efficiency by which HU and araC are transported into the cell—a pathway affected greatly by the medium conditions in certain cells, especially CHO cells (Downes & Collins, 1982; Downes, Johnson & Yew, 1983). We compared the effects of fresh and conditioned medium on TK cells pre- and post-u.v. irradiation and found no difference, which suggests that TK cells do not condition the medium so that it affects nucleotide transport. In order to eliminate the complication caused by the CHO cell-conditioning effect, fresh medium was always used during experiments. Therefore, the discrepancies in the drug inhibition of DNA synthesis are probably due to the differences in the endogenous pool sizes of the two cell lines. A simple method of measuring the relative pool sizes (Strauss, 1981) is to use various specific activities of $[^3]$H]thymidine to label the cells; the incorporation rate monitors the dilution of the isotope in the endogenous pool. Fig. 6 shows the incorporation of $[^3]$H]thymidine (20 $\mu$Ci/ml) in the presence of various amounts of cold exogenous thymidine into DNA of CHO and TK cells. The results demonstrate that in TK cells the isotope was effectively diluted away at an exogenous thymidine concentration of 10 $\mu$M, while in CHO cells the dilution was much less effective. TK cells should have a smaller

![Graph](image-url)
endogenous deoxyribonucleotide triphosphate pool than that of CHO cells if the transport mechanism and other factors are similar in both cell lines.

**The capacity for excision repair and the effect of inhibitors**

The capacity of TK cells to perform excision repair was assessed by nucleoid sedimentation. Fig. 7 showed the titration of nucleoid sedimentation against ethidium bromide. The biphasic curve indicated that the DNA in TK nucleoid is supercoiled (Cook & Brazell, 1975). When irradiated with 10 Jm⁻² followed by 30 min incubation, there was a reduction in the rate of nucleoid sedimentation probably associated with the relaxation of a number of supercoil loops by single-strand breakage. Two hours after irradiation, the nucleoid sedimentation is restored to control value. This biphasic change of sedimentation in relation to the incubation period after u.v. reflects the balance between incision near damaged sites and ligation of completed repair sites during excision repair (Cook & Brazell, 1976).

The production of single-strand breaks during excision repair is best resolved using inhibitors of DNA synthesis (HU and araC) as demonstrated by alkaline sucrose sedimentation (Fig. 8). Following alkaline lysis, DNA from cells u.v. irradiated and incubated with these inhibitors is found to contain single-strand breaks. The presence
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Fig. 7. The effect of u.v. radiation on the sedimentation of nucleoids. Nucleoids were prepared by lysing \(5 \times 10^4\) cells in 0·2 ml buffer (2·0 M-NaCl, 0·01 M-EDTA, 0·1% Triton X-100, pH 8·0) on top of a 4·6 ml sucrose gradient (15% to 30% sucrose, 2·0 M-NaCl, 0·01 M-EDTA, 0·002 M-Tris, pH 8·0), and centrifuged at 30,000 rev./min for 40 min at 20°C. The cells were generally labelled with \(^3\)H-thymidine, then irradiated with 9 J m\(^{-2}\) in PBS. After irradiation the cells were incubated in complete medium for 30 min (□—□) and 90 min (●—●). Sedimentation was performed with an unirradiated control (Δ—Δ) in the same run. Acid-precipitable material was counted for radioactivity. Inset: relative sedimentation rate of TK nucleoids in the presence of various amounts of ethidium bromide (EthBr) in the lysis buffer and sucrose gradient. The conditions for centrifugation were the same as above.

of breaks reduces sedimentation in alkaline sucrose gradients (Burg, Collins & Johnson, 1977; Collins et al. 1977; Collins, 1977). When TK cells were gently layered on an alkaline sucrose gradient and sedimented immediately, the velocity of the DNA peak migration was inversely correlated with the number of single-strand breaks. The results shown in Fig. 8 indicate that after 30 min of post-irradiation incubation, the amount of single-strand break accumulation depends on the type and concentration of the inhibitors. With u.v. alone, there was little change in DNA sedimentation, indicating few single-strand breaks, even when the dose was increased to 110 J m\(^{-2}\). Incubation with HU increased the amount of radioactivity in the top half of the gradient. araC by itself caused a more drastic reduction in sedimentation rate, while
Fig. 8. Rate of DNA unwinding during sedimentation in alkaline sucrose gradients. TK cells were preincubated with or without inhibitors for 1 h before u.v. radiation. After u.v. challenge the cells were incubated for 30 min in the presence or absence of inhibitors, approximately $5 \times 10^4$ cells were then lysed on top of an alkaline sucrose gradient and centrifuged immediately at 30 000 rev./min for 30 min at 4°C. Fractions were collected from the top and acid-precipitable material was counted for radioactivity. A, Unirradiated control; B, u.v. 11 J m$^{-2}$; C, u.v. 110 J m$^{-2}$; D, u.v. 11 J m$^{-2}$ with 0.01 M-HU; E, u.v. 11 J m$^{-2}$ with $10^{-4}$ M-araC; F, u.v. 11 J m$^{-2}$ with 0.01 M-HU and $10^{-4}$ M-araC.

the combined effect of hydroxyurea and araC retained the DNA peak near the top of the gradient.

Post-replication (daughter strand) repair

A pulse-chase experiment was performed to assess the ability of TK cells to carry out post-replication repair (Lehmann, 1981). After irradiation $^{14}$C-labelled cells were pulsed for 30 min with $[^3]$H]thymidine and chased for 1 h.

The results show that the behaviour of nascent DNA molecules in control and u.v.-irradiated cells was very similar at 0 h and after 1 h (Fig. 9), indicating that 13.5 J m$^{-2}$ of u.v. did not delay the ligation of the nascent species into bulk DNA; but 0.3 mg/ml caffeine delayed the chase rate slightly. The overall rate of incorporation of nascent DNA into the parent was faster than that in CHO cells at the same dose (Stamato, Hinkle, Collins & Waldren, 1981).

DISCUSSION

The DNA repair mechanism in fish cells has not been extensively studied. Of the
Fraction no. Fraction no. Fraction no.

Fig. 9. Post-replication repair of u.v. irradiated TK cells. Cells were generally labelled with $[^{14}C]$thymidine for 48 h. u.v. radiation (13.5 J/m$^2$) was carried out 4–6 h after washing off the $[^{14}C]$thymidine. The cells were pulse-labelled with $[^{3}H]$thymidine for 30 min immediately after u.v., then chased for 1 h with/without caffeine (0.2 mg/ml). About $5 \times 10^4$ cells were lysed on top of an alkaline sucrose gradient at 4°C for 12 h. The tubes were spun at 30,000 rev./min for 3 h at 4°C. Fractions were collected from the top, and acid-precipitable material was counted for radioactivity. A. Control, unirradiated, 30-min pulse, no chase. B. Control, unirradiated, 30-min pulse, 1-h chase without caffeine. C. Control, unirradiated, 30-min pulse, 1-h chase with caffeine. D. u.v. (13.5 J/m$^2$) irradiated, pulse, no chase. E. u.v. (13.5 J/m$^2$) irradiated, pulse, 1-h chase without caffeine. F. u.v. (13.5 J/m$^2$) irradiated, pulse, 1-h chase with caffeine. $^{14}C$ (○○○); $^{3}H$ (●●●). The arrow indicates λ DNA.

repair systems investigated, photoreactivation of the thymine dimer appears to be the most prominent, with a limited amount of excision repair activity (Mano et al. 1980, 1982; Regan et al. 1972). Here we have demonstrated that TK cells can undertake as much excision and post-replication repair as CHO cells.

Recent studies by Elliott & Johnson (1983) showed that excision repair activities were different in cells of different origin; they found that adult mouse kidney epithelial cells accumulated more single-strand breaks in the presence of HU and araC than kidney or lung fibroblasts. Gibson-D’Ambrosio, Leong & D’Ambrosio (1983) also demonstrated that cells cultured from various human foetal organs had different capacities for excision repair. All of the fish cell lines studied in different laboratories were fibroblasts originating from connective tissue (Regan et al. 1972), erythrophoroma (Mitani, 1983)
or fins (Cook & McGrath, 1967; Regan & Cook, 1967; Mano et al. 1980, 1982). Whether the excision repair activity found in TK cells is related to their epithelial nature and kidney origin remains to be investigated.

The cell survival and proliferation studies showed that the u.v. sensitivity of TK cells is comparable to that of HeLa and CHO cells (Downes et al. 1979; Collins, Downes & Johnson, 1980). The recovery of DNA synthesis after u.v. was, however, faster in TK than in CHO cells. Single-strand scission due to u.v. endonuclease cleavage was not apparent after u.v. irradiation except in the presence of inhibitors. As in other studies (e.g. see Collins & Johnson, 1981), HU and araC were found to differ in their capacity to accumulate single-strand breaks, while their combination resulted in the greatest accumulation of breaks.

We found that HU and araC were less effective in suppressing replicative DNA synthesis in TK cells than in HeLa and CHO cells. Cell survival depends, to a certain extent, on the supply of DNA precursors (Johnson & Collins, 1978; Yew & Johnson, 1979). The efficiency of the TK repair system in the presence of inhibitors might be explained if the internal pool of deoxynucleotides was considerably larger in TK than in CHO cells. However, the isotope dilution experiment shown in Fig. 6 revealed that the extent of $[^3]$H]thymidine incorporation was much more readily influenced by changes in specific activity in TK cells than in CHO cells. Thus we cannot claim that TK cells have larger DNA precursor pools than CHO cells. One possible explanation is that TK cells might have a very fast turnover rate of DNA precursors. This is at present under investigation.

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