Antagonists of DNA synthesis in corneal epithelial cells of the dogfish

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The epithelial cells of the cornea of the dogfish continue to incorporate thymidine into their DNA during incubation in elasmobranch Ringer's solutions under appropriate conditions. This incorporation of H3 thymidine can be markedly inhibited by including IUDR in the medium, or by preirradiation of the cells with gamma rays or UV light.

When the epithelial cells of the cornea of the dogfish are mechanically injured, a rapid incorporation of tritium-labeled thymidine (H3 thymidine) into deoxyribonucleic acid (DNA) takes place. Incubation in vitro of corneal epithelial cells of the dogfish also demonstrates a significant degree of incorporation of thymidine into DNA. The depression of thymidine uptake when the temperature is lowered (to 0° C.), or when oxygen tension is decreased, indicates a metabolic dependence.

The purpose of this study was to observe the effects of iododeoxyuridine (IUDR), ultraviolet (UV) light, and gamma-ray irradiation on the synthesis in vitro of DNA in corneal epithelial cells of the dogfish.

Materials and methods

The entire epithelial layer of the cornea of the dogfish (Mustelus canis) was removed with scalpel. Each incubation tube contained three entire epithelial cell layers in 5 ml. of elasmobranch Ringer's solution containing 100 μC (10^-3 M) of H3 thymidine. The tubes were incubated for 5 hours under a mixture of 95 per cent oxygen and 5 per cent CO2 at 20° C. In one group of experiments, varying concentrations of IUDR (from 10^-5 M to 10^-2 M) were added to the incubation flasks. Other groups of cells were exposed to 254 mJ of UV light (provided by a quartz immersion lamp at more than 30,000 microwatts per square centimeter) for periods of time ranging from 30 minutes to 4 hours prior to the addition of H3 thymidine. Incubations of irradiated and control cells were carried out for 5 hours with H3 thymidine in the incubation medium. The UV source was immersed in an 18 mm. test tube containing elasmobranch Ringer's solution and the suspended corneal cells. Temperature was maintained at 20° C. by suspending the entire assembly in a flowing water bath. A third group was exposed to gamma-ray irradiation in a dosage varying from 1,000 to 15,000 r (provided by a 137Cs source at 5,000 r per minute) prior to the addition of H3 thymidine. The 5 hour incubation with thymidine was then carried out. In two experiments, 125 I-UDR (10^-4 M) was added in place of the H3 thymidine. Tritium radioactivity was counted in a Packard

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liquid scintillation counter, and $^{125}$I was counted in a Nuclear-Chicago gamma-ray spectrometer.

When the incubation period was completed, the cells were frozen on dry ice, homogenized in, and washed five times with 5 per cent trichloroacetic acid (TCA) to remove all acid-soluble radioactivity. The TCA precipitates were dissolved in 0.1M NaOH, and aliquots were taken for the determination of radioactivity and DNA content.

DNA for chemical studies was extracted with sodium dodecyl sulfate and purified by means of Sephadex G 200 gel filtration. Ribonuclease (0.2 per cent in 0.05M PO$_4$ buffer, pH 7.0) was used to degrade the RNA for 4 hours at 45° C. in the detergent-extracted mixture of RNA and DNA prior to gel filtration. DNA was determined by the microindole procedure.

Ultracentrifugation studies on purified DNA (0.02 per cent in water) were performed at 42,040 or 50,000 r.p.m. in a model E Beckman-Spinco analytical ultracentrifuge with schlieren optics and a bar angle of 65°.

Experiments to determine the effect of heating the normal and irradiated DNA samples were performed by a method previously reported. The absorptions between 230 and 290 m$_4$ of 1 ml. aqueous solutions of DNA extracted from normal, 5,000 r gamma-irradiated, and 2 hour UV-irradiated cells were measured before and after heating with 17 per cent formaldehyde. Changes in optical densities were calculated at wavelengths at which maximum absorption took place.

Results

Fig. 1 summarizes the effects of increasing levels of IUDR on a H3 thymidine ($10^{-3}$M) incorporating system. Each point represents the average value of two determinations which agreed quite well. Thymidine uptake was depressed to 50 per cent of the control value by the addition of $10^{-4}$M of IUDR. When the concentration of IUDR was equivalent to that of thymidine ($10^{-3}$M), incorporation of thymidine was maximally depressed to less than 25 per cent of the control values. When $^{125}$I-UdR ($10^{-5}$M) was substituted for H3 thymidine in the incubation mixture, 7 per cent of the DNA thymidine was replaced by IUDR as measured by isotopic incorporation.

The effects of UV exposure on thymidine incorporation are shown in Fig. 2. Maximum inhibition (to 50 per cent of the control values) occurred following 2 hours of exposure.

![Graph](image-url)

**Fig. 1.** Effects of increasing concentrations of IUDR in the incubation medium on the in vitro incorporation of H3 thymidine (expressed as percentage of the control specific activities) into DNA. The thymidine concentration, as indicated in the figure, was $10^{-3}$M in all experiments. Incubations were carried out at 20° C. for 5 hours in elasmobranch Ringer's solutions.

![Graph](image-url)

**Fig. 2.** Effects of increasing exposure times of epithelial cells to UV light on the in vitro incorporation of H3 thymidine (expressed as percentage of the control specific activities) into DNA. Incubations were carried out at 20° C. for 5 hours in elasmobranch Ringer's solutions containing $10^{-3}$M H3 thymidine.

The uptake of thymidine into gamma-irradiated cells was maximally inhibited by 3,000 r exposure, resulting in a decrease to 50 per cent of the control values (Fig. 3).

Ultracentrifugation patterns of DNA from the control and the 5,000 r gamma-
Fig. 3. Effects of increasing gamma-ray dosages on the in vitro incorporation of H\textsubscript{3} thymidine (expressed as percentage of the control specific activities) into DNA of cornea epithelial cells of the dogfish. Incubations were carried out at 20° C. for 5 hours in elasmobranch Ringer's solutions containing 10\textsuperscript{-3}M H\textsubscript{3} thymidine.

Irradiated samples are shown in Fig. 4, A and B, and from the 2 hour UV-irradiated samples in Fig. 4C. The control DNA had a sedimentation rate of 46 S, and the gamma-irradiated DNA had 40 S, but the UV-irradiated DNA, being heterogeneous in size, had a sedimentation peak that was too diffuse to measure accurately.

The results of the formaldehyde heating experiments are shown in Fig. 5. The maximum increase in optical density (O.D.) and UV absorption shift occurred in the control DNA sample (0.201 units), as compared with an increase of O.D. of 0.020 units for the UV-irradiated DNA and 0.037 units for the gamma-irradiated DNA.

Discussion

Both UV light and gamma-ray irradiation, as well as thymidine analogue IUDR, have been shown to exert a marked inhibitory effect on in vitro (injury-induced) DNA synthesis in corneal epithelial cells derived from the dogfish. When the incubation media contained equivalent amounts of H\textsubscript{3} thymidine and IUDR (10\textsuperscript{-4}M), there was a 75 per cent depression of thymidine incorporation. Previous studies by others\textsuperscript{5,6} have indicated that the degree of in...
corporation of IUDR and thymidine should be similar when they are both present in equal concentrations. This effect indicates that, at high levels of IUDR, enzymes involved in DNA such as thymidylate kinase are markedly inhibited.7

A comparison of the results of the previous paper1 with those presented in this one shows that the effects of IUDR upon DNA synthesis depend very strongly upon the concentration present. At lower concentrations (i.e., $10^{-5}$M), the IUDR appears to be incorporated into DNA in a manner similar to thymidine. At higher levels (i.e., $10^{-3}$M), it is not only incorporated into DNA but also markedly inhibits DNA-synthesizing enzymes.

Exposure to UV and gamma-ray irradiation at their maximum doses resulted in a 50 per cent inhibition of thymidine incorporation. The UV absorption studies suggest that these forms of irradiation may exert their effects by breaking the DNA chains. The UV irradiation used appears to be more effective in breaking these DNA chains than the gamma-ray irradiation used on the basis of sedimentation rate and reaction to heating with formaldehyde. The UV absorption and ultracentrifugation studies suggest that these forms of irradiation may exert their effects by breaking DNA chains.

Heating the irradiated samples with formaldehyde gave much less of a hyperchromic effect than the control samples. This indicates an altered DNA from the irradiated cells which can no longer be denatured as can native DNA.

Further evidence of the breakage of DNA chains due to irradiation can be found in the lower sedimentation rates of the irradiated DNA sample.

REFERENCES