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Direct Evidence that Damaged DNA Results in Neoplastic Transformation — A Fish Story[1]

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ABSTRACT

SETLOW, R. B., AND HART, R. W. Direct Evidence that Damaged DNA Results in Neoplastic Transformation — A Fish Story.

There is convincing evidence for the hypothesis that DNA damage may result in malignant transformation. A specific test for the biological role of ultraviolet (UV)-induced pyrimidine dimers in DNA is photoreactivation (PR). If UV-induced tumor production is photoreversible, such a finding would indicate that dimers in DNA lead to neoplastic transformation. Fish were chosen for study because they contain large amounts of the PR enzyme. Portions of cell suspensions of tissue from various organs of the fish Poecilia formosa were exposed to UV radiation (254 nm) and were injected into isogenic recipients. The percentage of fish with tumors was determined 6 to 9 months later by gross pathology and histological examination. An incident fluence of 20 J/m^2 resulted in 10% of the fish with large granulomas and 100% with thyroid carcinomas. If the irradiated cell suspension was illuminated with PR light before injection, the yields of both types of lesions were reduced ~10-fold. If the PR light was given before the UV exposure, there was no reduction in the number of growths. These preliminary experiments indicate that pyrimidine dimers in DNA can lead to neoplastic transfromation.

Key words: *uv/pyrimidine dimers/photoreactivation*

INTRODUCTION

The international concern about environmental pollutants and their mutagenic and carcinogenic activities is reflected by the many experiments on the interaction between such agents and the macromolecular components of cells. Our interest in ultraviolet (UV) radiation as a carcinogenic agent stems from the large amount of basic photochemical and photobiological information concerning UV damage and its repair (1, 2) and from the fact that human skin cancer — which comprises over half of all the cancers of the white population of the United States (3) — is associated with UV radiation. Animal experiments indicate that it is the shorter wavelength component (< 320 nm) of sunlight that is the carcinogenic agent; since this component increases with decreasing latitude, as does skin cancer, it is reasonable to suppose that human skin cancer arises from such wavelengths in the sun's spectrum (4,5). It is noteworthy that one environmental impact of a large fleet of supersonic transports could be a decrease in the ozone level in the stratosphere with an attendant increase in UV radiation and skin cancer (6). We are interested in knowing whether DNA is the target macromolecule, and, if so, what initial molecular changes in DNA result in neoplastic transformation. None of the experiments to be described elucidate the mechanism by which such changes result in transformation.

Individuals with the disease xeroderma pigmentosum (XP) are a very high risk population for UV-induced skin cancer (7). The observation that most of these individuals are not able to repair DNA damage (2, 8) — they do not excise pyrimidine dimers from their DNA (9) — is evidence that DNA is the target and implies that damage to DNA is the causative agent. Moreover, a number of chemical carcinogens are also mutagens (10).

The damage some of them do to DNA is repaired by a sequence of steps that mimics the repair of UV damage in normal human cells (11). XP cells are unable to repair DNA damage from these carcinogens (11). This finding is further evidence that DNA is the target for neoplastic transformation.

Since UV makes many changes in DNA (1), we do not know whether the biologically important photoproducts are cyclobutyl-pyrimidine dimers or one of the many other alterations induced in DNA. The absence of dimer excision in XP cells is not conclusive evidence that dimers are the important causative agent, because other uninvestigated changes may not be repaired either. Just as for alkylation damage, the most common product may not be the most important biologically (12, 13). One particular repair system — enzymic photoreactivation (PR) — is specific for pyrimidine dimers (14, 15). Of the many products made by UV irradiation of DNA, only dimers are affected by PR. The process monomerizes the dimers, yielding the original unaltered polynucleotide, but leaves the other products untouched. Therefore PR can be used as a diagnostic tool. If formation of tumors by UV were prevented by PR, this finding would be evidence that cyclobutyl pyrimidine dimers are a lesion in DNA that results in neoplastic transformation.

A number of years ago Rogers (16) showed that if embryonic mouse lung tissue was irradiated in vitro with 254-nm radiation, it would give rise to adenomas upon transplantation to homologous recipients. Such experiments are attractive since the treatment of cells in vitro permits one to make good physical or chemical dosimetry measurements. However, since mice are deficient in PR activity (15), PR experiments cannot be used to assess the role, if any, of dimers in transformation. Marsupials do contain useful amounts of PR

enzyme (15); therefore in 1969 J. S. Cook, J. D. Regan, and R. B. S. designed experiments using marsupials. The experiments were not begun because of the associated housekeeping problems. Meanwhile, a letter in Science (17) led J. D. Regan to suggest the use of clones of fish as an experimental system. Fish cells contain large amounts of PR enzyme (15). Furthermore, the fish we used, Poecilia formosa, may be grown in clones, permitting successful transplantation of cells from one animal to another (18).

EXPERIMENTAL METHODS

Initial Experiments

We used several clones of the naturally occurring gynogenetic (nonsexually reproducing) fish Poecilia formosa (18, 19). The offspring of the fish are white and identical, and since the females are activated by the males of another species (black), the transmission of any male information is easily detected by the mottled skin color (20). The general experimental design is shown in Fig. 1. In the first series of experiments cells from larger (F-1) fish (1 year old, 5 cm long) were obtained from liver. The tissue was homogenized to yield cell clumps (3-8 cells per clump), the clumps were treated as indicated in Fig. 1, and 20 µl containing 3-5 X 10^5 cells was injected into the dorsal muscle of isogenic recipients (~3 to 4 months old). The recipients were scored for tumors ~6 months later by B. Koesner of the Veterinary Pathobiology Department, Ohio State University.

Later Experiments

In later experiments designed to improve the histological analysis, cells were obtained from younger fish (3-4 months, 2 cm long), treated as in Fig. 1, and injected into the abdominal cavity of recipients. In these experiments several tissues were homogenized together. Hence injected cells consisted of a mixture of liver, heart, and thyroid cells. The latter tissue represented ~40% of the injected cells. Fish were killed 6 to 9 months after the injection of cells and fixed in Bouin's solution. Tumors were scored by gross pathology and by the appearance of stained histological sections. We are deeply indebted to John Harshbarger of the Registry of Tumors in Lower Animals, Smithsonian Institution, Washington, D.C., for the histological analyses. It was he who first noticed that the fish injected with irradiated cells had thyroid carcinomas. His histological conclusions were confirmed by C. J. Dawe of the National Cancer Institute.

UV Irradiation and Photoreactivation

The UV radiation, primarily 254 nm, was obtained from a germicidal lamp. The incident fluence rate on cell suspensions containing ~2 X 10^7 cells/ml was 1.0 J/m^2/sec. The irradiated samples were ~1 mm thick, and the average incident fluence rate through the sample was ~0.1 J/m^2/sec. PR illumination was supplied by black light lamps in an incubator at 37°C. Typical PR fluence rates were 700 J/m^2/min, in the wavelength range 320-400 nm.

Carcinogen Treatment

Approximately 2.5×10^7 cells/ml in medium containing 10% fetal calf serum (to approximate the experimental conditions used to compare UV and carcinogen damage to human cells in ref. 11) were treated with the chemical carcinogen N-acetoxy-AAF (ref. 10) at concentrations of 10^{-5} and 10^{-4} M for 1 hr. At the end of this time the cells were collected by centrifugation and resuspended in Ringer's solution; then 20 μl was injected into isogenic animals.

RESULTS

Initial Results

The results obtained by injecting treated liver cells into the back muscle of fish have been described briefly (21). After an incident UV fluence of 20 J/m^2, ~10% of the fish had large tumors at the site of injection. Exposure of the cells to PR illumination after UV resulted in a large decrease in the numbers of tumors — a dose reduction factor of ~0.8. PR illumination by itself had little effect. Because such highly localized tumors were not suitable for histological examination, we turned to experiments in which treated cells were injected into the abdominal cavity.

Later Results

The initial attempts using cells injected into the abdominal cavity were not useful because of the presence of tuberculosis organisms in many of the fish. Many of the experimental animals contracted the disease and many had granulomas that might have been bacterially induced. After assuring that the colony was clean, we resumed the experiments.

For an authoritative opinion on infections and on tumors in the fish, we enlisted the assistance of John Harshbarger (see Experimental Methods). He observed that most of the fish injected with UV-irradiated cells had large numbers of noninfectious granulomas and, in addition, had thyroid carcinomas which were invasive into both soft and hard tissue. Thyroid carcinomas were present in 100% of the fish injected with 5×10^5 cells exposed to an average incident fluence of 10-20 J/m^2. Smaller or larger fluences gave fewer tumors. Table I shows the effect of PR illumination before and after the UV exposure. Note that PR after UV irradiation results in a very large decrease in the number of thyroid carcinomas, whereas illumination before UV does not. A similar finding is observed for the granulomas. Thus these data implicate pyrimidine dimers in DNA as the initial change that results in neoplastic transformation. (T-I)

Carcinogen-Treated Cells

Treatment of cells with N-acetoxy-AAF before injection resulted in the appearance of thyroid carcinomas in a large percentage of the fish, as shown in Table II. (T-II)

CONCLUSIONS

The initial experiments described here show that single exposures of fish cells to UV radiation result in neoplastic transformation, and that if the UV irradiation is followed by PR illumination, many fewer transformants arise. Our background knowledge of the photochemistry and photobiology (1, 14, 15) leads us to conclude that the initial change

in macromolecules resulting from the UV irradiation has been in DNA and, moreover, that the particular change in DNA has been the formation of pyrimidine dimers. The data, of course, say nothing about the mechanism(s) by which such changes are converted into neoplastic transformations.

It is instructive to estimate the probability of a transformation per dimer in cellular DNA. We estimate (from data in this paper and also unpublished) that an average incident fluence of 5 J/m^2 will result in approximately one transformed cell per 10^5 thyroid cells. This fluence converts ~0.02% of the thymines to thymine-containing dimers, which corresponds to ~1 dimer/10^7 daltons of DNA (9). Since there are about 10^{12} daltons of DNA per cell, there are 10^5 dimers/cell at 5 J/m^2. Hence the probability of a random dimer resulting in a neoplastic transformation is ~10^{-10}.

Studies on the magnitude of DNA repair following treatment of normal human cells with UV and with N-acetoxy-AAF indicated that 10^{-5} M AAF was approximately equivalent to 5 J/m^2 (ref. 11). In the experiments described here, 10^{-4} M AAF was equivalent to ~10 J/m^2. This comparison between measurements of repair in one species (human) and transformation in another (fish) may be farfetched, but the agreement isn't bad. We take these data as reinforcing the notion that damage to DNA is important to chemical carcinogenesis.

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TABLE I

EFFECTS OF PR ILLUMINATION[a] ON UV-IRRADIATED[b] CELLS[c] OF P. formosa

Treatment	Fraction of fish with granulomas	Granulomas per fish	Fraction of fish with thyroid carcinoma by	
			gross pathology	histology
UV	51/63	1.8	34/34	29/29
2.5 min PR + UV	49/57	2.0	26/26	22/22
5.0 min PR + UV	37/42	1.9	48/50	22/23
UV + 5.0 min PR	15/43	0.4	1/43	0/6

[a] 700 J/m^2/min (320-420 nm).

[b] 12 J/m^2 average incident fluence (254 nm).

[c] ~3 X 10^5 cells injected per fish.

TABLE II

EFFECT OF N-ACETOXY-AAF[a] ON CELLS[b] OF P. formosa

Concentration	Fraction of fish with thyroid carcinomas by	
	gross pathology	histology
0	0/50	—
10^{-5} M	14/50	—
10^{-4} M	45/51	10/10

[a] Treatment for 1 hr at the indicated concentration.

[b] $\sim 5 \times 10^5$ cells injected per fish.

FIGURE LEGEND

Fig. 1. Outline of an experiment to determine whether pyrimidine dimers in the DNA of irradiated cells result in neoplastic transformation. If they do, then there should be very few tumors in those fish injected with cells treated with UV plus PR compared with UV alone or compared with PR plus UV. The no-treatment and PR samples are controls.

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DO PYRIMIDINE DIMERS IN DNA RESULT IN TUMORS?

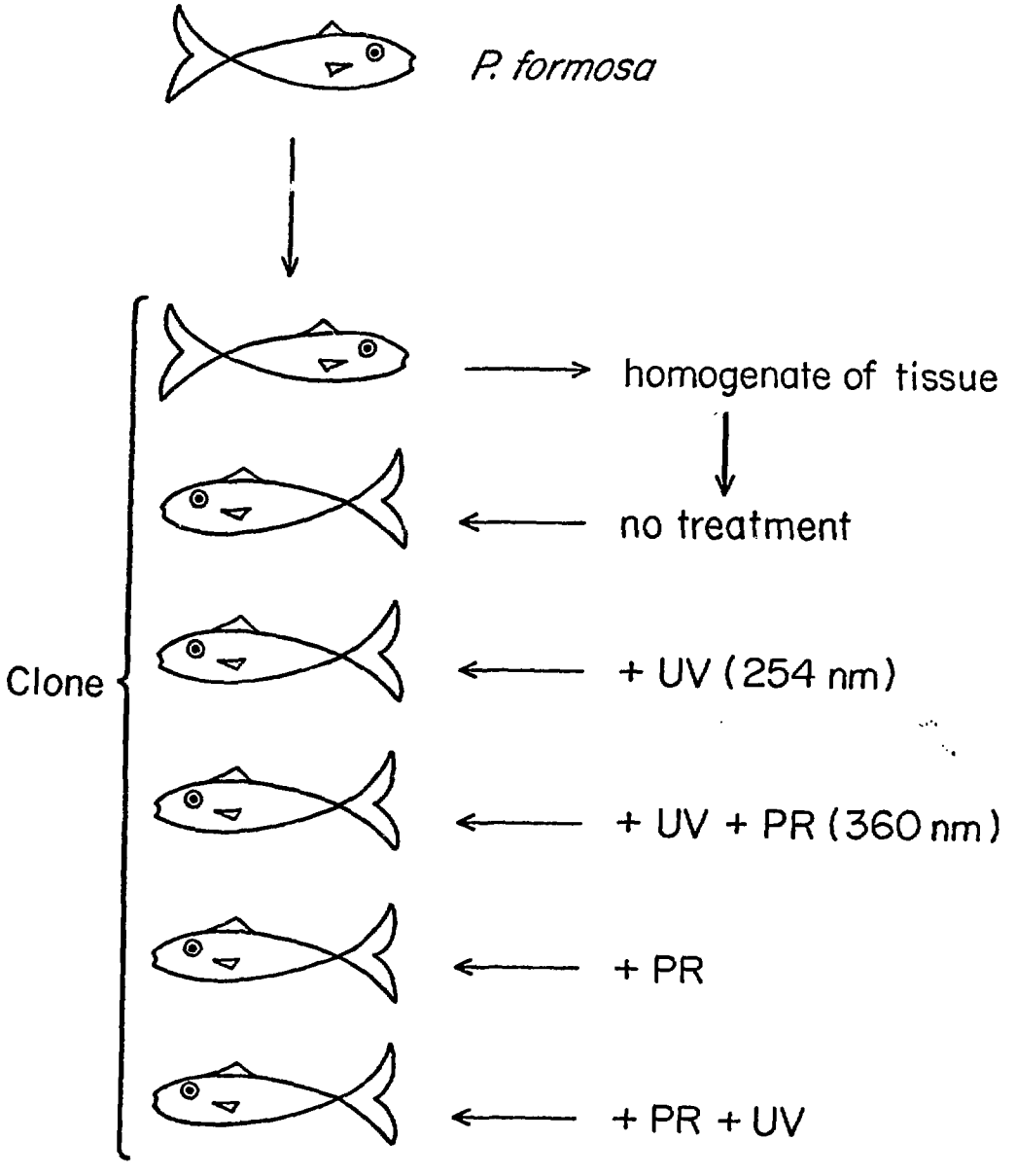

fig 1