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Evidence that pyrimidine dimers in DNA can give rise to tumors

(UV irradiation/photoreactivation/fish/thyroid)

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ABSTRACT **The Amazon molly, *Poecilia formosa*, is a small fish that grows in clones. Hence, cells from one animal may be transplanted to another without danger of rejection. Cells from thyroid and adjacent tissue were irradiated with UV light *in vitro* and injected into the abdominal cavity of isogeneic recipients. At appropriate UV doses and numbers of cells injected, all recipients showed exuberant thyroid proliferation. We give arguments and data indicating that the proliferation is a tumor, not a goitrogenic response. If the UV irradiation is followed, but not preceded, by photoreactivating illumination, the yield of thyroid growths is markedly decreased. Because other investigations have shown that photoreactivation monomerizes UV-induced cyclobutylpyrimidine dimers in DNA and does not affect other photoproducts, our data indicate that pyrimidine dimers in DNA can give rise to tumors.**

A number of physical and chemical environmental agents damage DNA *in vivo*. Many of these agents are carcinogenic (1–3), and various mutagen test systems show that there is an excellent correlation between carcinogenicity and DNA damage as measured by mutagenesis (4). Nevertheless, because treatment of DNA with carcinogens results in the formation of many products, it has not been possible to identify particular molecular changes in DNA as initiating carcinogenic events. To make such an identification, one needs an experimental trick that will change the relative proportions of the various products and permit observation of how tumor induction depends on these proportions. UV radiation makes many types of photoproducts in DNA. One of these—cyclobutylpyrimidine dimers (pyrimidine dimers)—has been shown to have lethal and mutagenic effects in microorganisms by virtue of the fact that such dimers can be monomerized by photoreactivating (PR) enzyme plus light of wavelength >320 nm (5, 6). Because, to the best of our knowledge, enzymic photoreactivation works only on pyrimidine dimers and not on other products, our finding of a PR effect strongly implicates pyrimidine dimers as the important initial DNA damage in tumor induction.

There is excellent evidence that most human skin cancer arises from solar UV radiation (7) and it is a good inference that DNA is the target molecule (8). Rogers (9) showed a number of years ago that UV-irradiated embryonic mouse lung would give rise to adenomas upon transplantation to homologous recipients. We have used another useful experimental system—the gynogenetic fish *Poecilia formosa*—to obtain data that indicate strongly that UV-induced pyrimidine dimers are involved in tumor formation. The animals contain PR enzyme (10) and may be grown in clones. Hence, one can treat cells *in vitro*, inject them into recipient animals, and score the recipients for tumor formation. We found that UV-irradiated cells from thyroid and adjacent tissue gave rise to thyroid tumors and that, if the irradiated cells were exposed to photoreactivating light (PRL) before injection, tumor induction was suppressed. Because PRL monomerized dimers in the DNA in cells (ref. 11; R. W. Hart, unpublished data), these observations connect dimers in DNA with tumor formation.

In preliminary reports of these results (12, 13), we described the tumors as thyroid carcinomas and, as a result of a series of misunderstandings, we incorrectly cited C. J. Dawe of the National Cancer Institute as confirming this conclusion. We deeply apologize to him for this incorrect attribution. In the present paper we present, in addition to quantitative data, our histological evidence for interpreting the exuberant thyroid growths as tumors.

METHODS AND MATERIALS

Animals. The clones of animals used in this study were originally collected from a freshwater pool near Brownsville, TX, by C. P. Haskins in 1946 and have been kept by him since that time. They were classified as to immunologically compatible types by Kallman and separated on this basis into four separate clones (14). C. P. Haskins generously supplied us with members of these clones in 1972. Several breeders were raised in individual 27-liter tanks containing three male black mollies. Resulting spawns were immediately removed, counted, and placed in 100-liter tanks at a density of ~1 per liter. After 2–3 months, these individuals were removed and placed in 470-liter tanks at a density of 0.3 per liter. The water contained 1 g of iodized table salt per liter and its temperature was maintained at 24° ± 2° with a photoperiod of 16 hr of light and 8 hr of dark.

Animals were fed twice a day with live baby brine shrimp and a high-protein meal containing the following (proportions in parentheses): beef hearts (30), tuna (15), fish roe (12), cooked spinach (6), marine fish meal (6), high-protein baby cereal (4), wheat germ (2), and iodized salt (1).

Cell Isolation and Injections. Donor animals (2–3 months old, 2 cm long) were placed on ice for 5 min and then given 3-min washes in each of 2.6% sodium hypochlorite, cold 95% ethanol, and sterile fish Ringer's solution. Scales were removed and tissue was excised from along the ventral aorta. The excised region contained thyroid, muscle, and cardiac tissue (from the thyroid gland, urohyal, and heart, respectively). Tissues were pooled and homogenized in fish Ringer's solution without Ca^{2+} or Mg^{2+} and containing isoniazid (0.7 mg/ml), streptomycin (1 μg/ml), and penicillin (1000 units/ml). Homogenization yielded primarily small clumps of two to five cells per clump. These were then treated as described below and 20-μl samples containing 2 to 5 × 10^5 cells were injected into the abdominal cavities of 25–100 isogeneic recipients (approximately 3–4 months old). We estimate that thyroid made up ~50% of the

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Abbreviations: PR, photoreactivating; PRL, photoreactivating light.

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Table 1. Thyroid tumor development depends on number of UV-irradiated cells* injected into isogeneic recipients of *P. formosa*

Cells injected, no.	No. with tumors†/ no. analyzed	% with tumors
4×10^5	19/20	95
2×10^5	25/40	62

* Cells from clone 4 animals were irradiated with an average 254-nm dose of 19 J/m^2.

† Clone 4 recipient animals were examined for gross lesions at 6 months after injection.

isolated tissue bed and that thyroid cells represented 10–15% of the injected cells.

Five to 10% of the animals died from the injection procedure. Of the remaining animals, >70% survived for 6–8 months, at which time they were killed. Because they were small, the bodies were fixed whole in Bouin's fluid. Fish that had died earlier were also fixed and analyzed. After fixation was complete the fish were dissected (with a razor blade) along the backbone into two halves. One half was kept. The second half was dehydrated and embedded in wax by routine histological procedures. Sagittal sections were cut at 8 μm, and the tissue was stained with hematoxylin and eosin. Lesions were scored by gross appearance and by microscopic examination of stained sections.

Irradiation Procedure. Cell suspensions at 4° were irradiated with 254-nm radiation from a low-pressure mercury arc at an incident dose rate of 1.1 W/m^2 measured by a Jagger meter (15); 1.9 ml of cell suspension as irradiated in 50-mm-diameter petri dishes (suspension depth, 1.9 mm). The optical densities of such suspensions were high, varying from 1 to 6 from experiment to experiment depending on the extent to which the suspension was depleted of blood and blood cells before irradiation. We have corrected the incident doses to give average doses through mixed samples (16) but the corrections may have large errors (17) and hence a strict dosimetric comparison from one experiment to another is not valid although comparisons within an experiment are valid.

PR treatment consisted of exposing cell suspensions at 30° to the light from six 15-W BL blacklights filtered through 6 mm of window glass to remove wavelengths <320 nm. The incident dose rate, as estimated by a Jagger meter (15), was 700 J/m^2·min.

After irradiation, the cell suspensions were centrifuged in a clinical centrifuge, the supernatant fluid was discarded, and the pellets were resuspended in Ringer's solution plus antibiotics so that there were 1 to 2.5 $\times$ 10^7 cells per ml.

RESULTS

UV Irradiation. In all experiments, exuberant thyroid growth was seen in most of the fish receiving a single injection of cells that had been exposed to UV radiation at an average dose of 10–20 J/m^2. The same findings were obtained with fish from clone 2 or clone 4, and there were no histological differences between clones in the appearance or extent of the resulting thyroid growth. The fraction of fish with thyroid growths depended on the number of irradiated cells injected (Table 1) and, for a fixed number of cells injected, on the UV dose (Fig. 1). There was an optimal dose range for obtaining a large response and so, in subsequent experiments, we injected 4 $\times$ 10^5 cells per animal and used UV doses in the optimal range. Most animals, including controls, also had noninfectious granulomas in the abdominal cavity and other tissues but their occurrence did not seem to be dose-dependent.

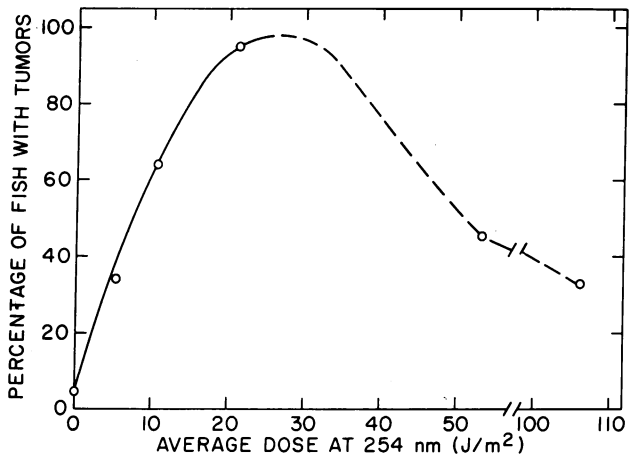

FIG. 1. Dose-response curve for tumor induction. Cells from clone 2 *P. formosa* were irradiated *in vitro* and 5 $\times$ 10^5 cells were injected into clone 2 recipients. Eight months later the animals were killed and 40–50 animals were scored for each point by gross examination for the presence of exuberant thyroid proliferation.

Thyroid Growth. In young untreated *P. formosa*, the thyroid gland consists of a few unencapsulated follicles scattered throughout the subpharyngeal connective tissue (Fig. 2). Exuberant thyroid growth in the experimental fish was readily apparent 6 months after the injection of cells. The growth consisted of a solid, smooth mass of tissue lying in the midline of the floor of the mouth, partially covering the anterior

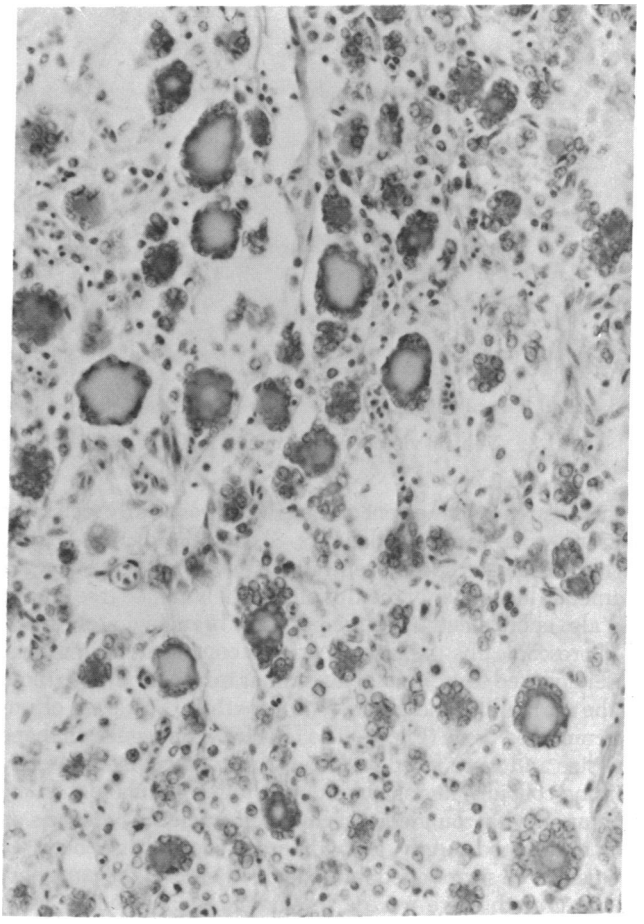

FIG. 2. Thyroid gland of young, untreated *P. formosa*, showing isolated thyroid follicles scattered among the subpharyngeal connective tissue. (H and E; ×266.)

FIG. 3. Ventral view of experimental molly, with operculae removed, to show the extent of the exuberant thyroid growths. (×14.)

branchial arches. The extent of the growth was clearly seen after the removal of the operculae (Fig. 3). In well-advanced cases, thyroid tissue had spread ventrally to enclose the first two gill filaments, leaving only the tips of the gill lamellae free, and it had also encroached upon the posterior branchial arches.

Microscopically, the thyroid growth consisted of numerous, closely packed thyroid follicles, similar in appearance to those of the normal thyroid gland. The growth had no areas of undifferentiated tissue (Fig. 4). The follicles were regular in shape and size and were filled with marginally vacuolated, deeply staining colloid. The follicular epithelium was cuboidal or low columnar with rounded, basally sited nuclei. There was an abundant blood supply, and numerous capillaries penetrated the tissue and surrounded individual follicles. At the periphery of the growth there were a few larger follicles with lightly staining, granular, eroded colloid. The thyroid tissue was highly invasive, and both muscle and bone were being eroded and destroyed. No metastases were found.

UV Irradiation plus PR Illumination. PR illumination given after the UV treatment resulted in a large decrease in the number of fish with thyroid growths, whereas PR prior to UV exposure had no effect upon the incidence of growths (Table 2).

Transplantation Experiments. Histocompatability tests (14, 18) have established that transplants of normal tissue from *P. formosa* within a clone (e.g., clone 4 → clone 4) survive indefinitely, but interclone transfers (e.g., clone 4 → clone 2) are rejected within a few weeks. Intraclone and interclone transplants were made with cells taken from a well-developed thyroid growth from a clone 4 fish that had been injected with UV-treated cells earlier. The experimental procedures (dissection, homogenization, and injection of the tissue) were the same as those used in the UV experiments. Four months later, 100% of the intraclone and interclone recipients themselves developed large thyroid growths. Intraclone transplants of normal thyroid tissue from old animals only rarely caused ex-

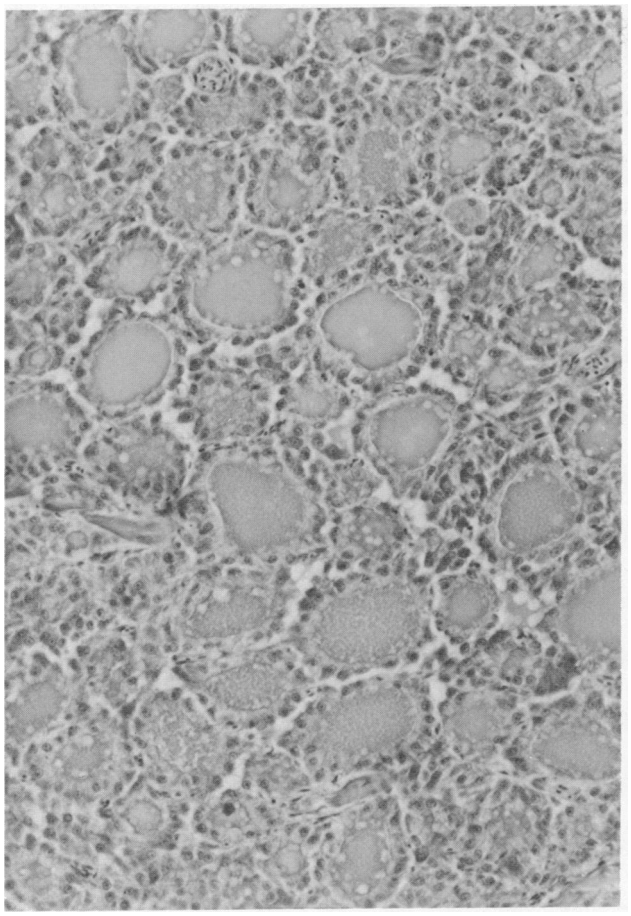

FIG. 4. Microscopic appearance of the thyroid growths in an experimental fish. (H and E; ×178.)

uberant thyroid growth in the recipient fish (Table 3). Our occasional finding of fish with exuberant thyroid growth after injection of normal thyroid tissue from old donor animals is not unexpected. Indeed, it could be foreseen from our surveys of the thyroid in old age, which showed that almost all senile fish spontaneously develop thyroid tumors (19).

DISCUSSION

We have shown that, when UV-irradiated cells of *P. formosa* are injected into isogeneic recipients, the recipients develop exuberant thyroid growths. Despite the fact that we have not observed metastases of these growths, we believe them to be neoplastic for reasons given in the following section. The fractions of animals and growths depend on the number of injected cells at a given UV dose and, for a given cell number, increases with dose up to ~20 J/m^2 of 254-nm irradiation, beyond which it decreases. Our interpretation is that the high doses cause extensive cell killing and such killed cells cannot be transformed.

Of the many molecular changes arising from UV irradiation of DNA, one of these—pyrimidine dimers—can be reversed by PRL. The observation that the number of tumors resulting from UV treatment followed by PRL is appreciably less than that from PRL followed by UV argues strongly that the initial lesions resulting in tumor formation are pyrimidine dimers in DNA. Such a conclusion, however, does not elucidate the molecular mechanism by which tumors arise from cells with damaged DNA, but it does permit us to make a guess at the probability that a random dimer will result in a tumor cell. The

Table 2. Effects of PR illumination of UV-irradiated cells* on the appearance of thyroid tumors

Treatment	Fraction of fish with thyroid tumors: Gross	Histologic
Experiment 1†		
UV (12 J/m^2)	34/34	29/29
2.5 min PRL + UV	26/26	22/22
5.0 min PRL + UV	48/50	22/23
UV + 5.0 min PRL	1/42	0/6
Experiment 2‡		
UV (24 J/m^2)	40/40	10/10
5.0 min PRL + UV	38/40	10/10
UV + 5.0 min PRL	0/22	0/10
Untreated	0/22	0/10

* Cells from clone 4 animals were irradiated with the average 254-nm doses shown. PRL was given before or after UV.
† 5×10^5 cells injected per animal.
‡ 4×10^5 cells injected per animal.

data in Fig. 1 show that 5×10^5 cells irradiated with 10 J/m^2 give rise, on the average, to one thyroid tumor (63% of animals with tumors). This dose results in 0.03% of thymine in dimers and hence gives rise to approximately 1 dimer per 5×10^6 daltons (20) and, because per cell there are 10^{12} daltons of DNA, there are 2×10^5 dimers per cell. If we assume that it is only thyroid cells (10^{-1} of the total) that can give rise to these tumors, the number of dimers injected per tumor formed is $(5 \times 10^5)(2 \times 10^5)(10^{-1}) = 10^{10}$. The reciprocal of this number, 10^{-10}, is the probability that the random dimer will transform a cell. If only a fraction, f, of thyroid cells reach the thyroid, the probability is $10^{-10}/f$.

Goiter vs. Neoplasia. In common with other animals, fish that have been kept for prolonged periods in water low in iodine or in poorly aerated water or have been fed diets containing antithyroid compounds frequently develop goiter. The inability of the thyroid to synthesize adequate amounts of thyroxine stimulates increased production of thyrotropic hormone by the pituitary gland which, in turn, evokes compensatory changes in the thyroid, including cell hypertrophy and hyperplasia.

There are difficulties in distinguishing histologically between goiter and neoplasia of the thyroid in all vertebrates; fish are no exception and, indeed, the diffuse nature of the gland compounds the problem. The growths seen in the *P. formosa* conceivably could be goiters, but several lines of evidence suggest that this is not the case. (*i*) The environment in which

Table 3. Effects of intraclone and interclone transplants of thyroid tumor* and normal thyroid† tissue of *P. formosa*

Type of transplant	Fraction of fish with thyroid tumors: Gross	Histologic
Clone 4 tumor to clone 4	10/10	10/10
Clone 4 tumor to clone 2	9/10	10/10
Clone 4 normal to clone 4	2/10	3/10

* 5×10^5 cells from tumor tissue were injected into each animal. Tissue was obtained from three 11-month-old clone 4 animals of Experiment 2 (Table 2) that had been injected with UV-irradiated cells 8 months earlier. Recipient animals were killed 4 months after injection.
† Normal tissue was obtained from ~14-month-old breeders; 5×10^5 cells were injected per recipient. The recipients were killed 4 months after injection.

the fish were raised was iodine-rich and aeration of the water was vigorous. (*ii*) Closely related Poeciliidae kept in the same aquarium showed no thyroid enlargement (21). (*iii*) Thyroid enlargement was entirely confined to those fish that had been injected with UV-treated cells or cells exposed to PRL before UV. The control groups (given untreated cells) or fish given cells exposed to PRL after UV showed no thyroid hyperplasia, although in an iodine-deficient environment goiters might have been expected to occur equally in all groups. (*iv*) These results were obtained in experiments with clone 2 and clone 4 fish, and the resultant thyroid growths could not be distinguished histologically. Both clones may be equally susceptible to goiter development, but this would seem somewhat unusual. Generally, there are distinct differences between strains of the same species in their response to iodine deficiency. (*v*) Our interpretation that the growth was a neoplasm was further strengthened by the results of intraclone and interclone transplantation experiments.

If it is considered that the thyroid growths seen in the experimental fish are goiters, the results of the transplantation experiments become difficult to explain. The cells of a goiter are untransformed cells, showing an adaptive but reversible response to excessive hormone stimulation. In interclone transfer experiments we would expect that goitrous cells would be rejected, as is normal tissue in interclone transfer (18). The fact that the transplanted cells induced vigorous thyroid overgrowth in interclone transfer argues that cellular transformation had occurred and that the original growth from which the cells were derived was a neoplasm.

Transplanted goitrous thyroid tissue can give rise to another goiter only when the hormonal status of the recipient fish is the same as that of the original host. If such an atypical hormonal condition occurred in all of our fish—due to a lack of iodine in the environment or to goitrogens in the diet—then it is curious that transplanted normal thyroid tissue did not inevitably induce thyroid growths in all of the recipients. Our results, from both the injection and transplantation experiments, are therefore consistent with the idea that neoplastic growth had been induced by the injection of UV-treated cells.

Unanswered Questions. Our results raise a number of questions that will have to be answered experimentally. (*i*) Why do irradiated cells give rise to extreme thyroid proliferation? Are the thyroid cells in the irradiated cell suspension the important ones for this effect? Experiments using ^{125}I-labeled cells show that injected thyroid cells end up mostly in the thyroid and not in other tissues (A. D. Woodhead, P. Scully, and R. B. Setlow, unpublished data). (*ii*) Will other irradiated cells such as liver and spleen give analogous results? (*iii*) Do the results depend on the site of injection? (*iv*) What is the earliest time after irradiation at which an interclone cell transfer will give rise to thyroid proliferation?

The initial phases of this work were done in the Biology Division of the Oak Ridge National Laboratory. We are grateful to James D. Regan of that Laboratory for the suggestion to use *P. formosa* as experimental material. We are also grateful to C. J. Dawe of the National Cancer Institute for stimulating discussions and advice. It is a pleasure to thank J. C. Harshbarger, Registry of Tumors in Lower Animals, Smithsonian Institution, for his many valuable suggestions and his continued interest in our work. Photomicrographs were taken by W. Marin, Jr. This research was sponsored jointly by the National Cancer Institute (Contract YOL-CP-50202) and the U.S. Energy Research and Development Administration.

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