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Promotion and Regression of Neoplasia by Testosterone-Promoted Cell Differentiation in *Xiphophorus* and *Girardinus*

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The precursors of the melanin-producing pigment cells of *Xiphophorus*, like those of other vertebrates, originate from the neural crest and migrate to their final location (2). They divide and undergo differentiation through the stages of chromatoblasts, stem(S)-melanoblasts, intermediate(I)-melanoblasts, advanced(A)-melanoblasts, melanocytes, and finally differentiate to melanophores, which are incapable of dividing. At a certain age the melanophores are removed by macrophages. Supply comes from S-melanoblasts. Depending on the genotype and the developmental stage of the fish, certain fish show a delay or even an arrest of differentiation in the stage of S-melanoblasts (1).

Studies on melanoma formation have shown that the only stage of differentiation in which the pigment cells are competent for neoplastic transformation is the stage of the I-melanoblasts (2). If a certain gene, the "tumor gene" *(Tu),* that mediates neoplastic transformation becomes derepressed by hybridization-conditioned elimination or mutation-conditioned impairment of *Tu*-specific regulating genes *(R),* the I-melanoblasts become transformed to TI-melanoblasts (T = transformed), which continue to differentiate to TA-melanoblasts, T-melanocytes, and finally T-melanophores, which are incapable of dividing.

Differentiation and division of the T-cells result in melanoma formation. The degree of malignancy of the melanoma depends on the ratio of incompletely differentiated, dividing T-cells to terminally differentiated, nondividing T-melanophores. The more incompletely differentiated T-cells are present in the melanoma, the higher is the degree of malignancy. Melanoma that consist predominantly of terminally differentiated cells are benign. The findings suggest that melanoma development, including initial tumor formation and further tumor growth, is a problem of cell differentiation rather than of cell division.

The objective of our experiments was to find out if substances that influence pigment cell differentiation may also influence melanoma formation. For this purpose we used the steroid 17-methyltestosterone, which was recently found to be a strong promoter of pigment cell differentiation in *Xiphophorus* (8,9).

This chapter contains part of the dissertation of A. Schartl.

To show a more general significance of our results, we used *Girardinus* besides *Xiphophorus* as experimental animals.

MATERIAL AND METHODS

Certain genotypes of the genera *Xiphophorus* HECKEL 1848 and *Girardinus* POEY 1854 (Pisces: Poeciliidae), which are all characterized by a derepressed *Tu* due to crossing conditioned elimination of *R* genes (see introduction), served as experimental animals. They can roughly be divided into (a) fish that had not developed melanoma due to a genetic and developmentally conditioned delay of pigment cell differentiation in the stage of the S-melanoblasts, and (b) fish that bore melanoma, which predominantly consisted of poorly differentiated transformed cells.

The genotypes out of which the experimental animals were chosen are as follows:

(a) *Xiphophorus Tu-Sp^e:* Backcross hybrids, produced by introgression of the *Tu*-containing gene complex *Tu-Sp^e* (spotted extended) of *X.maculatus* from the Rio Jamapa into the genome of *X.helleri* wild fish stock from the Rio Lancetilla (Mexico).

(b) *Xiphophorus Tu-Li (a/a):* Backcross hybrids, produced by introgression of the *Tu*-containing gene complex *Tu-Li* (Lineatus) of *X.variatus,* originating presumably from the Rio Panuco, into the genome of the albino *X.helleri (a/a).*

(c) *Xiphophorus Tu-Li (g/g):* Backcross hybrids like (b), however, nonalbino and homozygous for the golden gene *(g/g).*

(d) *Girardinus Tu-Vn:* Animals derived from *G. metallicus* wild fish stock from Cuba carrying the *Tu*-containing gene complex *Tu-Vn* (Ventral nigra), the origin of which is unknown.

All stocks were raised in our laboratory under standard conditions (25°C, 12-hr artificial light/24 hr) and were fed a standard diet (TETRA, Melle, FRG).

Testosterone Treatment

The fish were treated by adding an ethanolic solution of 17-methyltestosterone[1] (1 mg/ml; EGA-Chemie, Steinheim, FRG) to the aquarium water. The treatment was carried out continuously during periods up to 8 to 12 weeks and with different dosages ranging from 2 to 20 µl/l aquarium water per day. Adult females of the guppy *(Poeclia reticulata),* which are known to respond to testosterone by the development of male secondary sex characteristics, served as a test system for the biological activity of the hormone. Controls were kept under the same conditions and treated with the appropriate dosages of pure ethanol.

[1] 17 β-hydroxy-17-methylandrost-4-en-3-one

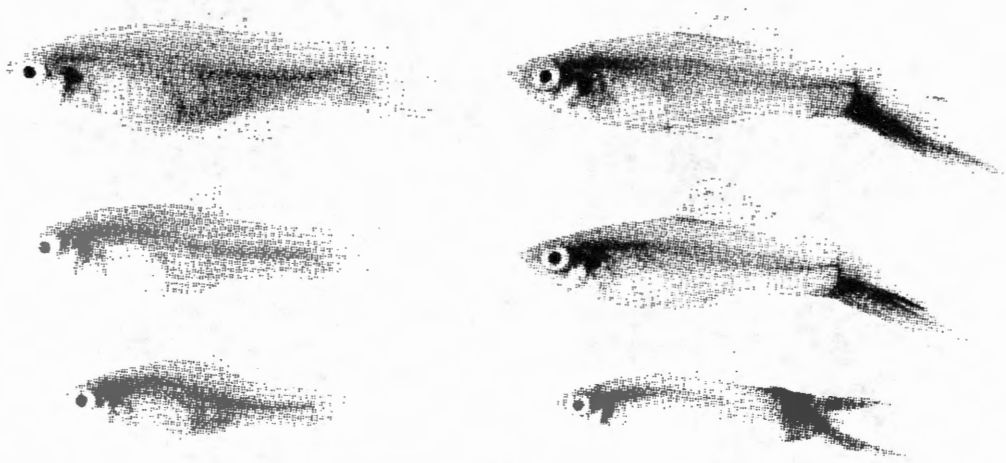

FIG. 1. 17-Methyltestosterone-induced malignant melanoma (2 μg/l aquarium water and per day). Untreated fish of *Xiphophorus Tu-Li (a/a) (left);* fish of the same genotype after 3 months of treatment *(right).*

Determination of Malignancy

The degree of malignancy of each melanoma was determined macroscopically and/or histologically according to the criteria given in ref. 2.

RESULTS

Tumor Promotion

In the first series of experiments we investigated the effect of testosterone on fish that, due to a delay of the differentiation of S-melanoblasts, had either not yet developed or normally do not develop melanoma.

In the *Xiphophorus Tu-Li (a/a)* an extremely benign amelanotic melanoma normally develops spontaneously at the age of 3 months on the side of the body. When treated continuously with testosterone[2] starting at birth, 72 out of 96 individuals (75%) developed at the age of 1 month malignant amelanotic melanoma. This melanoma, however, developed at the peduncle of the tail fin (Fig. 1). In the controls ($N = 104$) no melanoma was observed at that age.

In another experiment of this series, 317 animals of *Xiphophorus Tu-Sp*[e], which normally develop melanotic melanoma spontaneously at the age of 4 to 5 weeks on the whole body including the fins and occasionally the eyes, were treated from birth. All of the treated fish developed melanoma at the age of

[2] Further effects of testosterone, which will not be discussed in this chapter, were the development of male secondary sex characteristics in immature fish of both sexes and in adult females, and an increase of the expression of male secondary sex characteristics in adult males.

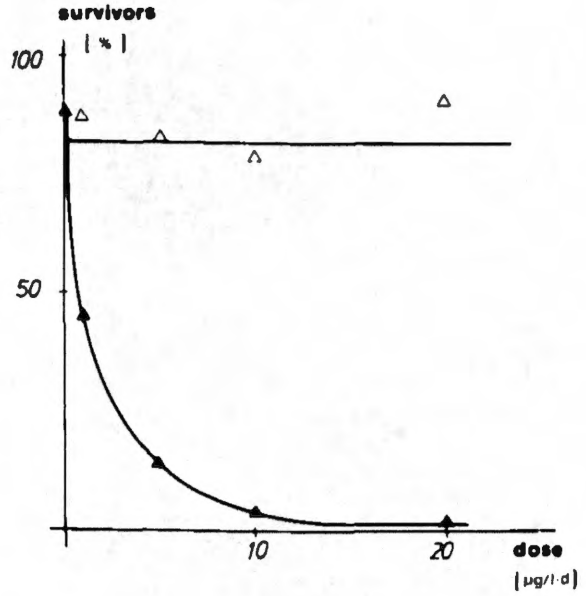

FIG. 2. Dosage dependency of cancer mortality in *Xiphophorus Tu-Sp*^{e} treated continuously from birth. Survivors after 3 weeks of treatment with 17-methyltestosterone: △: non-tumorous fish lacking *Tu-Sp*^{e}; ▲: fish carrying *Tu-Sp*^{e}.

3 days. The melanomas were extremely malignant and led to a testosterone dosage-dependent increase in cancer mortality as compared to the controls (Fig. 2).

In *Girardinus Tu-Vn,* melanotic melanoma develop normally in males older than 6 months after sexual maturation in the ventral region of the belly and of the operculi. Females of this genotype normally do not develop melanoma although carrying *Tu-Vn.* Testosterone-treated animals developed melanoma in 8-day-old fish of both sexes ($N = 20$) when treated from birth. Treatment of adult females ($N = 16$) resulted in melanoma formation within 5 to 7 days. In about 6 weeks the melanomas reached a phenotype similar to that of untreated males. The growth and malignancy of these melanomas were enhanced by further testosterone treatment, and these exceeded the spontaneous melanoma of untreated males (Fig. 3). Adult tumor-bearing males showed only a slight increase of neoplasia following treatment.

Tumor Regression and Tumor Suppression

In the second series of experiments, those fish that bore the type of melanoma that consisted predominantly of poorly differentiated transformed cells were treated. In 162 out of 208 individuals (78%) of those adult fish of *Xiphophorus Tu-Sp*^{e} which bore malignant melanoma on the whole body, the amount of terminally differentiated T-melanophores was increased after 3 to 4 weeks of testosterone treatment. The promotion of cell differentiation resulted in a benignization of the melanoma. T-melanophores were removed by macrophages, thus leading to tumor regression in the fins, the abdominal part of the body, and

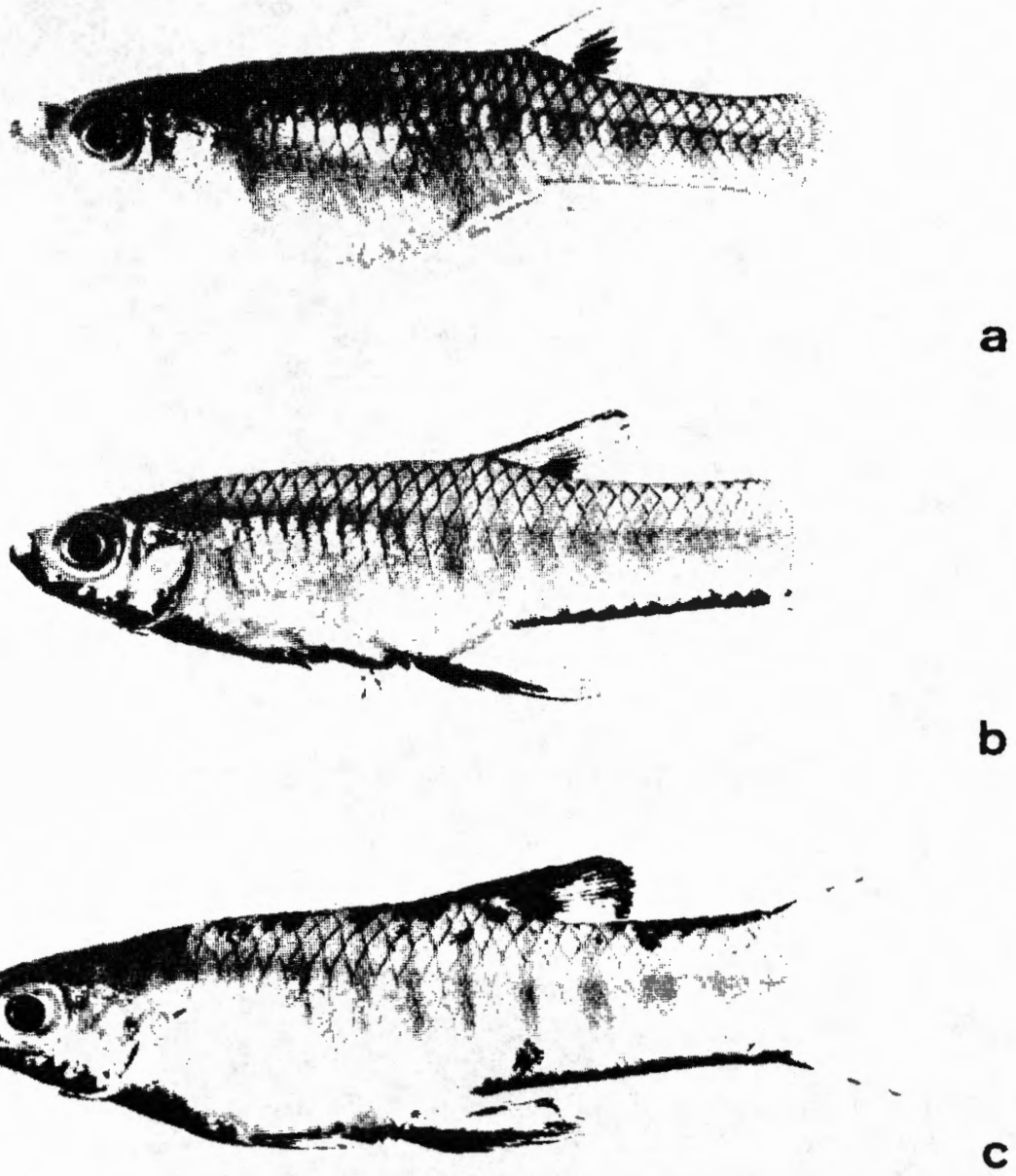

FIG. 3. 17-Methyltestosterone-induced melanoma (20 μg/l aquarium water and per day). **a:** Untreated female of *Girardinus Tu-Vn;* **b:** female after 6 weeks of treatment; **c:** female after 3 months of treatment.

the caudal part of the trunk. No regression was observed in the peduncle of the tail. After a further treatment of 3 months, all tumors were reduced to small benign melanoma in the peduncle of the tail (Fig. 4). In controls, 17 of 198 animals (8.6%) showed a regression of the melanoma, which, however, was never as prominent as in the treated fish.

In *Xiphophorus Tu-Li (g/g),* normally benign melanotic melanoma develop spontaneously in adults at the age of 4 months on the side of the body. If these animals ($N = 24$) were treated from the onset of melanoma formation,

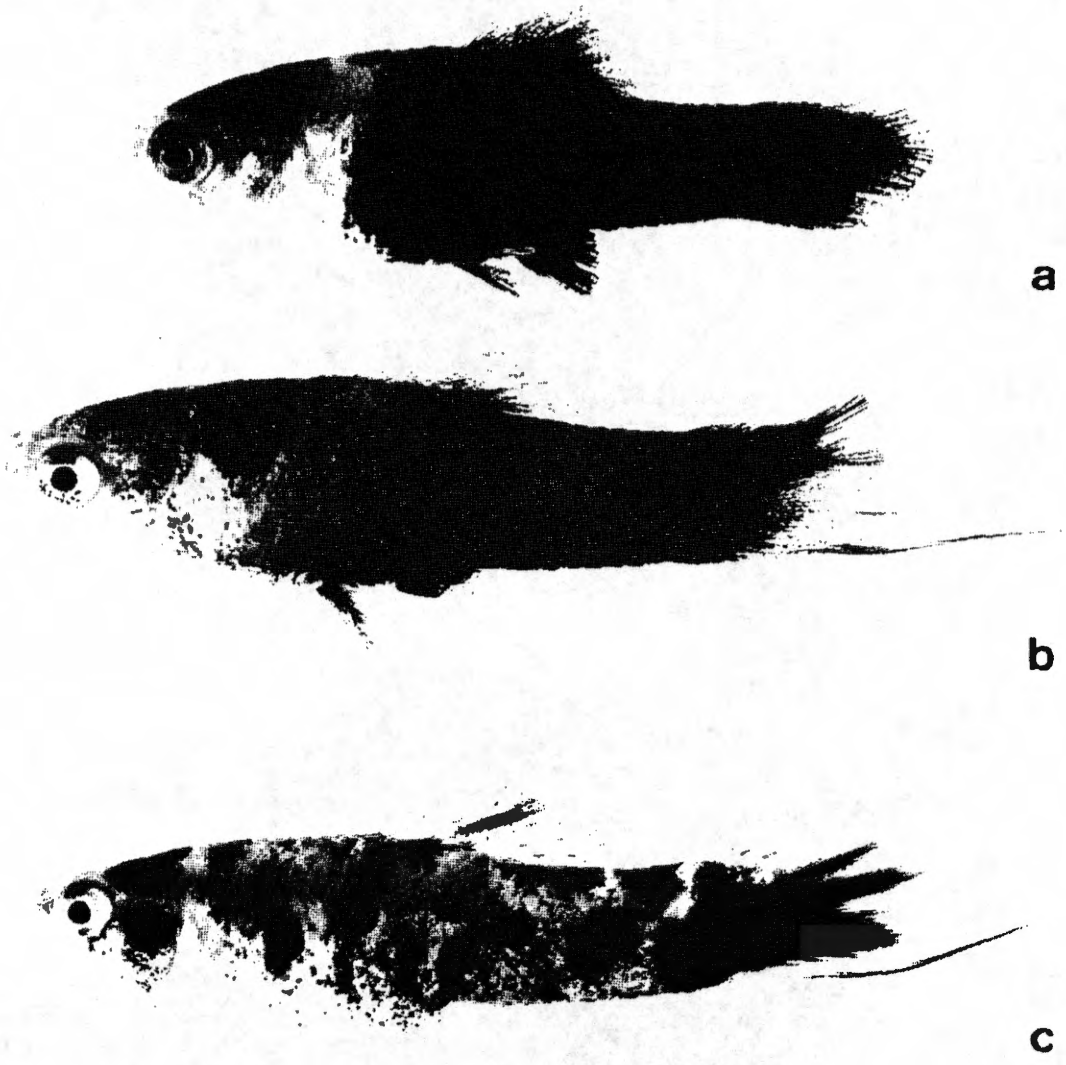

FIG. 4. Regression of malignant melanoma following treatment with 17-methyltestosterone (2 μg/l aquarium water and per day). **a:** Untreated fish of *Xiphophorus Tu-Sp*^{e}; **b:** same animal after 3 weeks of treatment; **c:** same animal after 8 weeks of treatment.

they developed only small T-cell colonies consisting of terminally differentiated T-melanophores. In these fish the formation of melanoma was suppressed (Fig. 5).

DISCUSSION

In one group of the *Xiphophorus* genotypes including *Girardinus,* testosterone has promoted melanoma formation, and in the other group it has suppressed melanoma formation. The dual effects can be explained by a promotion of cell differentiation mediated by the steroid hormone (9).

In one group, the majority of the pigment cell precursors are arrested in a

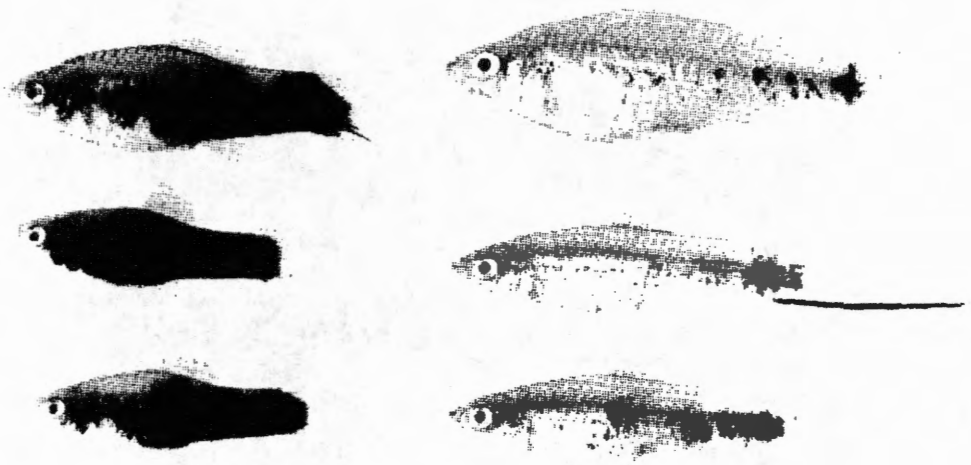

FIG. 5. Suppression of melanoma formation following treatment with 17-methyltestosterone (4 μg/l aquarium water and per day). Untreated fish of *Xiphophorus Tu-Li (g/g) (left);* fish of the same genotype after 3 months of treatment *(right).*

precompetent stage (see introduction) and may become competent via testosterone-promoted differentiation. Concerning melanoma development, two different situations have to be considered: First, melanoma does not yet develop because the majority of the pigment cells is arrested in the precompetent stage. Under the influence of testosterone, they differentiate to the competent stage. Thus testosterone appears to be an inducer of melanoma. Second, melanoma formation starts because some of the precompetent cells reach the competent stage due to an endogenous promotion of cell differentiation. Under the influence of testosterone, an increased amount of these cells reach the competent stage. In this case, testosterone appears as an enhancer of melanoma growth.

In the other group, the majority of the pigment cell precursors reach the competent stage for neoplastic transformation autonomously, and melanoma develops. Due to the influence of testosterone, the incompletely differentiated T-cells complete differentiation. The terminally differentiated T-cells that are incapable of dividing are removed by macrophages. Thus testosterone (a) may convert malignant melanoma to a benign phenotype, (b) may cause regression, and (c) may even prevent tumor formation. Testosterone, therefore, appears in these fish as a tumor suppressor.

According to the initiation-promotion model of carcinogenesis proposed by Berenblum (4), 17-methyltestosterone can be considered a tumor promoter or cocarcinogen. In the experiments presented here, "initiation" is represented by the derepression of *Tu,* whereas "promotion" is represented by the testosterone-induced differentiation of cells from a precompetent stage to a stage where the derepressed *Tu* can become active. Thus initiation means a genetic change, namely derepression of *Tu,* whereas promotion means an epigenetic shift in the stage of differentiation of the cells.

On the basis of our results one can also explain the cocarcinogenic effect of "classic" tumor promoters like the 12-O-tetradecanoylphorbol-13-acetate (TPA) (5) as well as the cocarcinogenic effect of substances such as diethylstilbestrol (11), many androgens (6), and retinoids (10), which are also known to promote cell differentiation. These compounds also require a genetic change as a precondition for their cocarcinogenicity. We assume that the genetic change may correspond to the derepression of *Tu*, whereas the cocarcinogenicity may correspond to the differentiation-promoting effect of methyltestosterone in our experiments.

Androgens, retinoids, and other compounds have also shown both carcinogenic and anticarcinogenic activity in certain experiments (3,7). We assume that the dual effect corresponds to that of testosterone reported here. Our results, including the findings on cocarcinogenic and anticarcinogenic effects of the compounds mentioned above, suggest that depending on the stage of differentiation of the target cells, "classic" tumor promoters like TPA may also act as anticarcinogens.

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