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Short Contribution

Hormonal factors from the mammalian pineal gland interfere with cell development in *Hydra*

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ABSTRACT A partially purified, melatonin-free low-molecular-weight extract from the ovine pineal gland with antitumor activity (YC05R), interferes with terminal differentiation in the interstitial cell line of *Hydra*. Nematoblasts developed into defective nematocytes that were subject to cell death and the tentacles eventually became devoid of nematocytes. In an attempt to identify the causative components of the extract, several known potential constituents were assayed. Two factors were found to have similar effects, although only in rather high concentrations: 1α, 25 dihydroxyvitamin D_3 (>150 nM) and pinoline (>5 μM), a natural tryptophan-derived β-carboline. The proliferative activity in the interstitial cell line was only slightly reduced by these factors. Two other β-carbolines that occur in the mammalian brain, harmine (10 μM) and n-butyl-β-carboline-3-carboxylate (β-CCB), caused the premature death of epithelial cells and thus the development of dwarfish animals which, however, continued to generate new animals by budding. The pineal extract probably contains some more, still unidentified components that interfere more potently with cell development, in *Hydra* as well as in mammals.

KEY WORDS: *pineal gland, 1alpha, 25 dihydroxyvitamin D_3, pinoline, harmine, Hydra*

There is growing evidence that the mammalian pineal gland has antitumor activity attributable only partially, if at all, to the known pineal hormone melatonin. A partially purified, melatonin-free extract obtained by ultrafiltration from the pineal gland, termed UM05R, caused a reduction of the cell population in several tumor cell lines (Bartsch *et al.*, 1987,1992). The extract was assayed with mammalian cells. We now tested a similar extract, termed YC05R, on *Hydra*. We chose this animal because it belongs to a basic animal phylum and is considered a perpetual, immortal embryo, for it unceasingly replaces cells of advanced age and cells having fulfilled their tasks by substitutes generated from stem cells. In *Hydra,* the pineal extract appeared to act in a manner similar to an extract from *Hydra* prepared and tested by Fujisawa (1988), in that both extracts reduced the population of certain cell types (nematocytes). Since pineal extracts are at present not available in quantities that allow classical, assay-guided purification of the components, we tested some known, potential low molecular weight constituents of pineal extracts for their capacity to evoke similar responses in *Hydra*.

Loss of nematocytes after treatment with pineal extract

Eighty *Hydra* polyps were daily exposed to an extract YC05R from ovine pineal glands that contains molecules in the range of 500-1000 Da. After eight consecutive days of treatment, the animals were unable to catch and ingest food because the tentacles lacked any stinging cells (nematocytes), and the animals appeared to be partially anaesthetized. The tentacles devoid of nematocytes preserved their normal shape, remaining long and slender. The animals were still able to regenerate a head or foot. However, wound closure was considerably delayed. After a transverse cut the wounds are normally closed in two hours; they remained open for 12 to 24 h in the YC05R-treated animals.

A second and third experiment was accompanied by a microscopic examination of the inventory of nematocytes in the tentacles, by a quantitative examination of the cellular composition of the body column (Fig. 1), by an analysis of the proliferative activity in the various cell lineages (Table 1), and by a quantitative evaluation of cell death in these lineages (Table 2).

Hydra is composed of two main cell lineages: (1) epithelial cells and (2) the interstitial cell line that produces nematocytes and nerve cells. Interstitial cells are sometimes found as single cells but occur predominantly in nests comprising 2, 4, 8, 16 or 32 cells. Cells found as single cells or occurring in pairs are considered to be multipotent stem cells or neuroblasts (David and Campbell, 1972; David and Gierer, 1974). Since true stem cells and early neuroblasts cannot be distinguished with certainty at present, they are collectively termed stem cells in this study. Interstitial cells that

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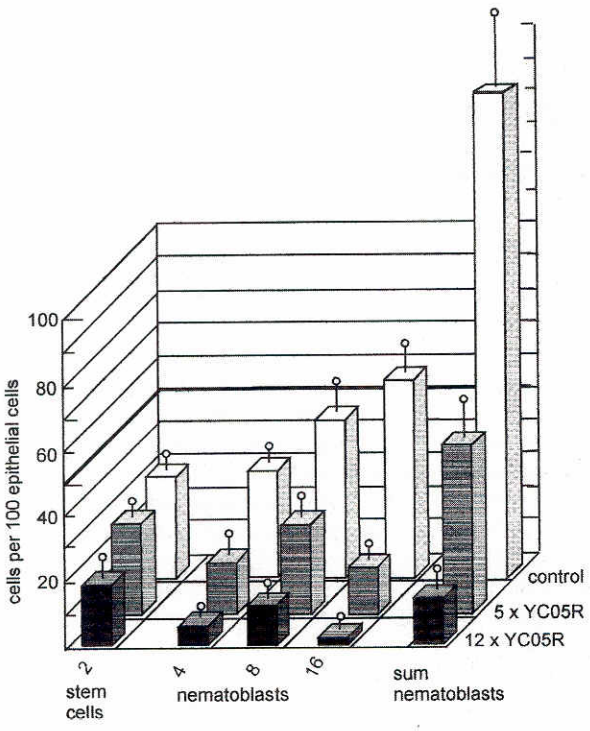

Fig. 1. Relative abundances (%) of interstitial cells per 100 epithelial cells in YC05R-treated and in control animals. *The animals were treated for four hours daily on 5 or 12 successive days. Then each animal was separately macerated on a slide (Method of David, 1973). An area comprising 300 to 2000 of epithelial cells was evaluated per slide. The values from several slides were taken to calculate means ± standard deviation. Statistical significance was tested by the parametric unpaired t test. The reduction in the number of nematoblasts is significant, the reduction in the number of stem cells is not.*

are found in groups of four and more cells are nematoblasts. If they do not yet contain capsules and their number is below 32, they potentially can divide. This is shown by their ability to incorporate the thymidine analog BrdU.

After 5 and after 12 days of treatment with YC05R, a sample of 5 to 10 polyps was removed and analyzed. Microscopic examination of dissociated cells and quantification of cell death frequencies pointed to a YC05R-induced disturbance of the terminal differentiation in the I-cell lineage. The nematoblasts did not develop functional capsules. Nematocytes with distorted capsules were observed in the subtentacular body column and in the proximal section of the tentacles. They were subject to cell death (Table 2) and prematurely sloughed off or removed through phagocytosis by epithelial cells. Therefore, the tentacles were gradually deprived of nematocytes. The sequence in which the various types of nematocytes in the tentacles disappeared, reflects their normal abundance: first the stenoteles were lost, then the more numerous isorhizas, finally the abundant desmonemes (similar to the photographically documented sequence shown in Lange *et al.*, 1990). After 9 to 12 days the animals were unable to feed and the experiment was finished.

The quantitative analysis of the cellular composition of the animals combined with BrdU labeling of proliferating cells confirmed disturbances in the advanced interstitial cell lineage. The number of nematocytes and nematoblasts decreased (Fig. 1), although the proliferative activity in the stem cells was only slightly reduced (Table 1). Frequently, differentiating nematoblasts contained fragmented DNA in their nuclei (Table 2). Main cause of the loss was premature cell death.

The temporal sequence in which members of the interstitial lineage were lost appears to be opposite to the sequence observed when the interstitial cell lineage is eliminated by destroying the stem cells. Hydras devoid of interstitial cells - termed epithelial hydras - lack stem cells, nematocytes and nerve cells, and are motionless. Such epithelial hydras have been produced by applying colchicine (Campbell, 1976), hydroxyurea (Bode, 1983), lithium ions (Hassel and Berking, 1988), and by means of extracts from *Hydra* containing an unidentified low-molecular-weight active component (Fujisawa, 1988). This *Hydra* extract reduced the number of stenoteles by preventing precursor cells from entering the stenotele pathway.

The nematocyte cell line can also be eliminated by pulsed exposure of hydras to ammonia (Lange *et al.*, 1990), and in this case the temporal pattern of cell loss was similar to that observed after application of the pineal extract. Therefore, it cannot be excluded with certainty that the destruction of nematocytoblasts is caused by a rapid enzymatic release of ammonia from components of the extract. On the other hand, premature cell death in the interstitial cell lineage was also observed after pulsed application of vitamin D_3, a nitrogen-free potential constituent of the extract, but not after applying many of the nitrogen-containing, putative constituents.

Loss of nematocytes caused by pinoline and 1α,25 dihydroxyvitamin D_3

At present, pineal extracts are not available in quantities that allow classical, assay-guided purification of the causative compo-

TABLE 1

S-PHASE LABELING INDEX DETERMINED BY THE BRDU/ANTI-BRDU METHOD

Treatment	interstitial cells			
	epithelial cells	nests of 2	nests of 4	nests of 8
Control	30 ± 7	58 ± 17	74 ± 8	70 ± 9
YC05R 0.6 mg/ml	26 ± 14	49 ± 21	61 ± 14	61 ± 14
1,25D3 24 nM	33 ± 9	60 ± 18	73 ± 8	67 ± 10
1,25D3 240 nM	26 ± 13	**48 ± 11**	**60 ± 10**	**60 ± 10**
Pinoline 1 µM	37 ± 12	79 ± 8	84 ± 6	65 ± 7
Pinoline 10 µM	27 ± 9	**44 ± 18**	**59 ± 9**	n.d.
Harmine 10 µM	27 ± 11	53 ± 14	68 ± 10	n.d.

The animals were treated for four hours daily on five successive days. After the last treatment 5 to 10 animals from each group were incubated in 5 mM BrdU overnight (18.00 - 9.00). Immediately after incubation each animal was separately macerated on a slide. Samples comprising 300 to 1800 of epithelial cells and 10 to 40 I-cell nests of each type were counted per slide, and the labeling index (= percentage of labeled cells or nests) determined. The values from several slides were taken to calculate means ± standard deviation. Statistical significance was tested by the parametric unpaired t test as well as by the non-parametric one-sample chi-squared test or the NxK chi-squared test. Bold: significant differences from the corresponding control values. n.d. = not determined

nents. Therefore, we tested known constituents of pineal extracts which might also be present in YC05R, even only in trace amounts. Eventually two components were found that had destructive effects on the differentiation of nematoblasts similar to those of the pineal extract. These components were 'pinoline', one of several β-carbolines with putative hormonal functions that are generated in the pineal gland, and '1α,25 dihydroxyvitamin D_3' (1.25 $(OH)_2D_3$). As with the pineal extract, the proliferative activity, as examined with BrdU, was only slightly reduced by pinoline or 1.25 $(OH)_2D_3$. The loss of nematocytes was caused by premature cell death. However, the doses necessary to eliminate nematocytes from the tentacles were rather high: >1 μM for pinoline and >100 nM for 1.25 $(OH)_2D_3$.

Loss of epithelial cells caused by β-carbolines

Two other β-carbolines that are known as plant alkaloids but are also found in the mammalian brain, 'harmine' and 'n-butyl-β-carboline-3-carboxylate (β-CCB)', caused a shrinkage of the animals to dwarfish sizes. Apparently, the balance between cell proliferation and cell loss was disturbed. When the experiment was finished after 12 days of treatment, the size of the polyps had been reduced to that of freshly detached normal buds. Nevertheless, the dwarfish animals continued to produce new animals by budding, though these were tiny when they detached, indicating a disturbed balance between cell birth and cell death even in young animals.

Conclusions

The proliferative activity, as examined with BrdU, was only slightly reduced by 1.25 $(OH)_2D_3$ or the β-carbolines (Table 1). On the other hand, nematoblasts and epithelial cells were detected with fragmented DNA (Table 2). Therefore, it is concluded that both the loss of nematocytes and the dwarfish size were due to premature cell death rather than reduced proliferative activity of the progenitor cells.

TABLE 2

CELL DEATH FREQUENCY (TUNEL LABELED CELLS)

Treatment	nematocytes per tentacle per tentacle	nematoblasts in nests of 4 percent of total	nematoblasts in nests of > 4 percent of total	epithelial cells percent of total
Control	8 ± 7	none	none	0.5
YC05R 1 mg/ml	**290 ± 190**	none	**25**	2
1,25D3 24 nM	3 ± 2	none	none	0.5
1,25D3 240 nM	**28 ± 10**	none	8	4
Pinoline 1 μM	**17 ± 5**	none	none	none
Pinoline 10 μM	**490 ± 300**	none	**34**	5
Harmine 10 μM	8 ± 8	none	3	**9**
b-CCB 10 μM	12 ± 9	none	6	**8**

The animals were treated for two hours per day on five successive days. In the last two days the animals were starved. Twenty hours after the last treatment the heads and feet were removed, the remaining gastric columns macerated and the isolated cells fixed on slides. The heads with the adhering tentacles were fixed and examined as whole-mounts. The slides were processed using the standard protocol from Boehringer. For whole-mounts all recommended incubation times were prolonged. The data are given as number of labeled cells per tentacle or percentage of all cells observed, and were compared by means of the non-parametric Mann-Whitney U-test. Significant differences (at 5% level or better) are given in bold.

A common denominator of all these factors that cause premature cell death might be their genotoxic potential (De Meester, 1995). Quantitative considerations make it likely that the pineal extract contains components that interfere with cell development more potently than ammonia or those putative components of pineal extracts tested in this study.

Vitamin D_3 or derivatives of it might well participate in the control of the cellular composition in *Hydra*. Natural β-carbolines are heterocyclic, dehydrogenated derivatives of tryptophan that are found not only in the ovine pineal gland and other mammalian tissues but also in plants and sponges (Airaksinen and Kari, 1981; Ichiba *et al.*, 1994). However, whether β-carbolines are regular hormonal factors in *Hydra* is unknown at present.

Experimental Procedures

The ovine pineal extract YC05R was prepared by I. Maidonis following a protocol published in Noteborn *et al.* (1988). The extract was applied in a concentration containing 0.33 mg dry mass per ml of culture medium. Eighty *Hydra magnipapillata*, strain wt 105, were daily exposed to the extract four hours after feeding for two hours. Similarly, solutions of substances purchased from Calbiochem, Sigma or RBI-Biotrend were administered for two hours daily. When ethanol or DMSO had to be used to prepare stock solutions, the controls received the same amount of solvent (maximum 0.1%).

The following potential components of the extract were tested: melatonin, glutamine, glutamate, adrenaline, noradrenaline, histamine, dopa, dopamine, serotonin, GABA, folic acid, vitamin D_3, 1α,25 dihydroxyvitamin D_3, leukotriene C_4 and D_4, prostaglandin F_{2a}; E_2; J_2; lipoxine A_4, isoxanthopterine, 2,4 pteridinediol, antineoplaston (3-phenylacetylamino-2,6-piperidinedione); harmalol, harmanol, harman, harmaline, harmine, pinoline (6-methoxy-1,2,3,4,-tetrahydro-9H-pyrido-(3,4b)indole, also termed 6-methoxy-1,2,3,4-tetrahydro-β-carboline) and n-butyl-β-carboline-3-carboxylate (β-CCB).

In addition, the following synthetic pharmaceutical β-carbolines, purchased from RBI-Biotrend, were tested: ethyl-β-carboline-3-carboxylate (β-CCE); methyl-β-carboline-3-carboxylate (β-CCM); N-methyl-β-carboline-3-carboxamide (FG-7142); methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). Data are only given for compounds which evoked particular effects and not merely symptoms of general toxicity.

Cell proliferation was examined with the BrdU/anti-BrdU method (Plickert and Kroiher, 1988) after the animals had been incubated with BrdU overnight, because the S-phase maximum is at midnight (David and Campbell, 1972). BrdU-labeled cells were detected using the alkaline phosphatase-based immunohistochemical procedure offered by Boehringer Mannheim (kit No1 758 756). Cell death was quantitatively determined in whole-mounts and macerates of 2-day starved animals with the TUNEL method using the *in situ* cell death detection kit No. 1 684 795 provided by Boehringer, Mannheim.

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