The fate of larval chondrocytes during the metamorphosis of the epibranchial in the salamander, *Eurycea bislineata*

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**SUMMARY**

The metamorphosis of the epibranchial cartilage, a skeletal component of the hyobranchial apparatus, in the salamander *Eurycea bislineata* entails a combination of the reabsorption of a larval cartilaginous element with the simultaneous genesis of an adult cartilage in the same place. In this study we focus on the fate of the larval chondrocytes. Two hypotheses are considered: one, larval cells simply die off during metamorphosis, or, alternatively, they dedifferentiate and participate in the formation of the adult element.

Thyroxine treatment and experimental tissue manipulation coupled with measurements of thyroxine levels using radioimmunoassay show that, within 24 h after T4 treatment, larval chondrocytes in the epibranchials exhibit large autophagocytic vacuoles, disruption of the rough endoplasmic reticulum, abnormally shaped mitochondria, abundance of lysosomes and nuclear degeneration, all symptoms of the onset of cell death. In conclusion, evidence from light microscopy, TEM and SEM show that the larval chondrocytes in response to rising levels of thyroid hormones undergo a process of lysosomal autophagocytosis and do not participate in the formation of adult structures.

**INTRODUCTION**

Since the early experimental work by Gudernatsch (1912), Allen (1918, 1925) and Etkin (1932) (see also reviews by Dodd & Dodd, 1976, and Gielbert & Frieden, 1981), amphibian metamorphosis has been perceived as the differential response of the various larval tissues to global cues, usually fluctuating levels of hormones. Some tissues can undergo dramatic rearrangement, or even complete degeneration, while others remain essentially unchanged. As part of an ongoing study of the organization of cartilage metamorphosis in amphibians, we report some results addressing the fate of larval chondrocytes in skeletal elements which degenerate during metamorphosis. There are two possible hypotheses: one, the chondrocytes in the degenerating larval element dedifferentiate, return to a mesenchymal condition, and participate in the development of adult structures. Alternatively, larval cells, in response to some global cue, undergo cell death.

*Key words: Eurycea, thyroxine, cartilage, metamorphosis.*
Our study has focused on the metamorphosis of the hyobranchial apparatus of the salamander *Eurycea bislineata* (Fig. 1), and, in particular, on what is perhaps the most puzzling event of this system: the simultaneous degeneration of the larval first epibranchial cartilage and the formation of the adult epibranchial in almost the same place (Figs 2 and 3).

*Eurycea bislineata* is a lungless salamander (family Plethodontidae) abundantly represented along the streams of the eastern United States. *Eurycea* and its allies are characterized by a complex life cycle in which the hyobranchial skeleton is not only morphologically different between the larva and the adult but it is involved in different functions. *Eurycea* larvae are adapted to an aquatic existence. The larval hyobranchial skeleton (Fig. 1A), and its associated musculature, is involved inbranchial respiration and suction feeding. In primitive (generalized) adult salamanders, as well as frogs, the hyobranchial skeletal elements and muscles are the dominant effectors of pulmonary ventilation (De Jongh & Gans, 1969; Severtsov, 1972), but in the lungless plethodontid salamanders, including *Eurycea*, the adult hyobranchial apparatus forms a highly sophisticated feeding mechanism involving tongue projection (Fig. 1B) (see Lombard & Wake, 1976, 1977; Wake, 1982; Ozeti & Wake, 1969; Regal, 1966; Severtsov, 1972; Thexton et al. 1977). Thus, the hyobranchial skeleton has to undergo significant changes during
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metamorphosis to adapt to its new function in a terrestrial environment. The work of Smith (1920; reviewed in Wilder, 1925) provides a fairly detailed histological analysis of the transformations involved in the development and metamorphosis of the hyobranchial apparatus in *Eurycea bislineata*. Our observations have confirmed Smith’s conclusions summarized in Fig. 1. As mentioned above, and the subject of this paper, Smith’s (1920) major discovery was that the adult epibranchial is not homologous to the larval first epibranchial (some authors, e.g. Romer & Parsons (1977, p. 212), refer to the epibranchials as ceratobranchials; here we follow Smith & Wilder’s nomenclature). Fig. 3 clearly illustrates the degenerating larval cartilage, next to the forming adult element. We present the results of experiments that show that in response to raising levels of thyroid hormones the chondrocytes of the first larval epibranchial undergo cell death and autophagocytosis.

MATERIALS AND METHODS

Animals

Over 250 specimens of *Eurycea bislineata* from Massachusetts and New Hampshire were used in this study. Larvae were maintained at a density no greater than 5–7 animals per litre of
Fig. 3. Photo of the gill arches (ventrolateral view) of the cleared and stained specimen illustrated in Fig. 2 (squared area). The adult epibranchial (E) is developing next to the larval first epibranchial (e1). The matrix of e1 is degenerating (arrow). Larval epibranchials 2 (e2) and 3 (e3) do not appear affected yet.

50 % Holtfreter’s solution with 0·1 g l⁻¹ MgSO₄.7 H₂O added. Adults were kept in plastic boxes with moist paper towels.

**Microsurgery**

While still in a premetamorphic condition, 22 larvae were anaesthetized using a 1:500 concentration of ethyl m-aminobenzoate and prepared for microsurgery which was accomplished using finely sharpened dissecting scissors and watchmaker forceps. Open branchial clefts assured premetamorphosis and allowed for easy access to the underlying epibranchials in the hyoid apparatus. Depending on the specimen, portions of the left first epibranchial were removed to serve as controls. After the operation the animals were placed directly into separate bowls containing ice-cold modified Holtfreter’s to which streptomycin and penicillin had been added. We experienced a 100 % postoperative survival rate. After 24 h at 4°C, L-thyroxine (3,3',5,5'-tetraiodo-L-thyronine) (Sigma Co., St. Louis, Mo.) was added to the Holtfreter’s solution in the concentration of 5 × 10⁻⁸ M. Animals were then returned to the 15–18°C room where they remained for periods ranging from 24 h to 18 days. Bowls were cleaned and fresh T₄ solution was added daily. At predetermined times the animals were again prepared for surgery as above. The corresponding right epibranchial was removed and placed in fixative. Following surgery, animals were sacrificed and fixed in 10 % neutrally buffered formalin, to be subsequently prepared for histology to confirm that the correct epibranchial had been removed.

Six specimens were used as controls, i.e., one larval epibranchial was removed but they were not placed in thyroxine but preserved at intervals corresponding to the treated specimens. In those cases no changes in the morphology of the remaining larval epibranchials were observed. Various skeletal elements were removed from naturally metamorphosing individuals to compare their cellular morphology with T₄-treated individuals.
Histology

All specimens used in the experiments were either cleared with trypsin and KOH and stained in toto with alcian blue 8GX (for cartilage matrix) and alizarin red S (for calcium deposits) (Hanken & Wassersug, 1981), or embedded in paraplast and sectioned serially at 10 μm. The sections were stained with modified tricromate Heidenhain's Azan technique (Baldauf, 1958). In addition, to study the process of normal metamorphosis, 214 specimens at various stages of metamorphosis were cleared and stained and 28 were serially sectioned.

All the skeletal elements that had been removed in vivo were placed immediately into 3-0 % glutaraldehyde in 0-1 M-sodium cacodylate buffer, pH 7-3, with 7 % sucrose. The tissues were left in this fixative for 2 h at 4°C. After rinsing in a series of cacodylic buffers for an hour at room temperature, the tissues were postfixed in 2 % OsO4 in 0-1 M-cacodylic buffer for 1 h at room temperature and then stored in buffer at 4°C until embedding. Tissues were dehydrated in ethanol, infiltrated with propylene oxide and embedded in Epon 812 after Luft (1961).

Transmission and scanning electron microscopy

Ultrathin sections were obtained using a Sorvall MT2B ultramicrotome; sections were stained with uranyl acetate and lead citrate. 1 μm sections were also obtained and, after heat fixing to glass slides, were stained for ten seconds with 0-5 % toluidine blue in saturated sodium borate. Thin sections were examined and photographed in either a Zeiss 9-S2 or an RCA EMU-4 transmission electron microscope.

For scanning electron microscopy, specimens, fixed as indicated above, were bisected using a razor blade, dehydrated in ethanol, and critical-point dried using a Tousimis SamDri PVT.3 drier. Specimen stubs were then sputter coated with gold-palladium and viewed and photographed using an AMRAY 1000 scanning electron microscope.

T3 and T4 radioimmunoassay

Radioimmunoassay (RIA) techniques to measure T3 and T4 levels in plasma serum were modified after Larsen (1976) and Regard et al. (1978). Slight modifications of the procedure were required to increase the sensitivity of the assay when dealing with small serum volumes. 2 μl aliquots of Eurycea serum were used for the T4 assay, while 5 μl aliquots were used in the T3 assays. No serum from different animals was pooled, and assays were run in duplicate (T3) and triplicate (T4) unless prevented by limitations in the volume of blood available from a given specimen (the amount of plasma serum that can be obtained from a single Eurycea ranges from 5 to 30 μl). The reported results are the average of the three counts. Adult Eurycea serum was used for the standard curve for the T4 assay while serum from the newt Notophthalmus viridescens was used in the T3 assays. The serum from adults of these two species was found to contain levels of T3 and T4 below the sensitivity of our assay. Levels of non-specific binding proteins were tested for every individual and used in the calculation of true % binding. In general, the difference between the non-specific binding levels of the standard animals and the experimental animals was negligible. Using these procedures, the minimum detectable levels were estimated to be 0.13 μg 100 ml−1 for T4 and 40 ng 100 ml−1 for T3.

RESULTS

Table 1 summarizes the number of specimens treated, the number of days of exposure of the animal to thyroxine (in all cases the specimens had the left first epibranchial removed, to be used as a control, prior to immersion of the specimen in the thyroxine solution), as well as the recorded serum levels of L-thyroxine (T4) and L-triiodothyronine (T3). Albeit limited by the low number of observations, the RIA data on thyroid hormone levels indicates that the T4 in the external
environment becomes rapidly incorporated by the organism. Larval specimens do not have measurable levels of T₃ or T₄ in their serum (Alberch & Gale, unpublished data). However, as early as 24h after immersion in the T₄ solution, we record high levels of T₄ in the serum (12–18 pg/μl⁻¹). The T₄ serum concentration was found to be even higher at 48h (25–32 pg/μl⁻¹), before decreasing and levelling off to levels ranging between 1·5 to 8·5 pg/μl⁻¹. All specimens analysed exhibited detectable levels of T₄. One assay of T₃ was run after 24h of exposure to exogenous T₄ showing no detectable levels of the T₃ in the serum, indicating that if T₄ was present in the serum it had not either been transformed into the T₃ form or triggered the production of T₃ by the thyroid yet. After this initial lag relative to T₄ levels, T₃ exhibits a similar pattern, which is characterized by an initial rise to high levels (3 pg/μl⁻¹ at 3 days) followed by a decline and subsequent levelling off.

Some changes in the morphology of the chondrocytes were observed as early as 24h after immersion in T₄. These changes became more accentuated 48h after T₄ treatment (Fig. 4). The treated larval chondrocytes appear highly vacuolated, with deformed nuclei (Fig. 4A,B), in contrast to the normal appearance exhibited by the chondrocytes in the control side (Fig. 4C). Unlike the larval chondrocytes, the perichondral cells do not appear to be negatively affected by the thyroxine treatment (Fig. 4A). In fact, autoradiographic labelling with [³H]thymidine indicates that perichondral cells respond to the treatment by increasing their rate of cell proliferation (Alberch & Gale, in prep.). As exposure to thyroxine continues, the signs of larval chondrocyte degeneration increase. Fig. 5 illustrates some chondroblasts prior to T₄ treatment and a cell after 11 days of treatment. The described cellular changes occur prior to any signs of metamorphosis in external morphology. It also appears that the process of cell death is autonomous, in the sense that cells began undergoing autophagocytosis while still in their lacunae. Matrix degeneration and tissue reabsorption occur much later in the process. In our studies, particularly after the fourth day of T₄ treatment, all the cells in the

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*Number between parentheses indicates number of specimens with RIA measurements.
larval epibranchial exhibited some degree of vacuolization. After 7 days, and in many cases earlier, a majority of the chondrocytes were dead, i.e., the nucleus had degenerated and the cytoplasm was effectively absent.

The sequence of morphogenetic events triggered by exogenous T₄ treatment closely resembles the naturally occurring metamorphic events. This conclusion is
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based on the histological and ultrastructural analysis of over 70 naturally metamorphosing animals. In Fig. 6 we illustrate a representative example of an advanced stage of normal metamorphosis, where the adult epibranchial \((E)\) is undergoing chondrogenesis, while the cells in the larval element \((e)\) appear highly vacuolated. Note that there are still no obvious signs of degeneration in the matrix of the larval element. TEM photographs show the typical chondroblastic morphology of the cells forming the adult element (Fig. 7). These cells are characterized by long cytoplasmic processes, extensive rough endoplasmic reticulum as well as matrix vesicles, which suggests active synthesis of extracellular matrix components. At this early stage of chondrogenesis the ECM is still sparse. In contrast, the chondrocytes from the larval epibranchial exhibit clear signs of cell degeneration and death (Fig. 8). Many autophagic vacuoles and lysosomes, coupled with the absence of an organized rough endoplasmic reticulum and the presence of swollen mitochondria, are all signs of autophagocytosis. Many other larval cells, both in experimental and in normally metamorphosis animals, showed

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Fig. 6. Cross section of a naturally metamorphosing specimen illustrating the larval epibranchial \((e_1)\) and the adult epibranchial \((E)\) undergoing chondrogenesis. Arrow shows the position of the hypertrophic chondrocyte photographed in Fig. 8. Bar equal to \(10\,\mu\text{m}\).

Fig. 5. (A) Normal larval chondrocyte in SEM. \(cm\), cartilage matrix; \(l\), lacuna; \(e\), cartilage cell. (B) Larval chondrocytes that have recently divided and are secreting extracellular matrix \((ecm)\). (C) Cross section of a normal larval chondrocyte; \(n\), nucleus. (D) Cross section of a larval chondrocyte exposed to \(T_4\) for 11 days. Note vacuoles \((v)\). Bars equal to \(5\,\mu\text{m}\).
condensations of nuclear heterochromatin as well as degeneration of the nucleus, also suggestive of cell death.

CONCLUSIONS

Our results unequivocally show that the fate of the larval chondrocytes of the first epibranchial cartilage in *Eurycea* is cell death. We have no evidence that the larval cells can dedifferentiate and participate in the formation of the adult cartilage. These results support the view of development as an irreversible process of restriction of fate.

Alberch & Gale (in prep.) have measured, using radioimmunoassay techniques (RIA), the levels of T₃ and T₄ during the normal metamorphosis of *Eurycea*. In agreement with previous studies (e.g. see Larras-Regard et al. 1981 and review by White & Nicoll, 1981), neither the larvae nor the adults have any detectable levels of these thyroid hormones. Metamorphosis in amphibians is characterized by a rapid increase of T₃ and T₄ in the serum. During the metamorphosis of *Eurycea*, Alberch & Gale (in prep.) recorded serum levels ranging from 2 to 18 pg μl⁻¹ for T₄ and from 0-5 to 2 pg μl⁻¹ for T₃. Animals exposed to exogenous levels of T₄ exhibited an initial surge of endogenous serum T₄ that was almost three times the concentration measured in normal metamorphosis. This initial concentration of 25–32 pg μl⁻¹ decreased to levels more in the normal range (7-5 to 8-5 pg μl⁻¹) in
the subsequent days of treatment with T4 (see Table 1). These results indicate that the exogenous hormones penetrate into the tissues of the organisms as early as during the first 24 h. This observation is consistent with the results reported by Robinson et al. (1977). These authors, studying how much of the T4 enters the tail tip of the metamorphosing anuran Xenopus in vitro, found that the amount of T4 in the tail tips increased during the initial 24 h to subsequently remain constant.

The reason for the detected initial 'surge' of hormone levels is not clear, since simple diffusion of the exogenous T4 into the serum could not explain, in the face of constant exogenous concentrations, the subsequent decrease and 'levelling off' of the hormones to within about one-third of the initial concentration. The observed T4 pattern could be the result of a combination of diffusion of exogenous hormone and the onset of endogenous synthesis of T4 by the thyroid followed by a decline in the plasma levels due to an increase in the rate of T4 binding to larval tissues. As discussed by Galton (1983) and Larras-Regard et al. (1981), there is no evidence to suggest that deiodinated T4 is converted into T3 in amphibians. Robinson & Galton (1976) suggest, based on their in vitro experiments, that T4 has direct biological activity in amphibians. Our data tend to support their conclusions, since after 24 h of treatment, we observe significant morphogenetic

![Fig. 8. TEM of a larval chondrocyte cell indicated with an arrow in Fig. 6 showing large vacuoles (v) with cellular debris (d), poorly organized rer and abnormal mitochondria (m). Many electron dense lysosomes and matrix vesicles are also apparent. Bar equal to 1 μm.](image-url)
responses in the absence of any detectable levels of T₃. T₃ exhibited a similar surge after 3 days of exposure to T₄. We favour the hypothesis that tissue binding is the parameter that regulates the levels of these thyroid hormones in the serum. This is supported by the observation that specimens that are allowed to metamorphose immersed in a constant T₄ solution exhibit extraordinarily high concentrations of T₄ in their serum (25–50 pg ml⁻¹) relative to controls that exhibit undetectable levels at the end of metamorphosis (Alberch & Gale, unpublished). This pattern might reflect the continuous diffusion of the exogenous hormone into the organism coupled with a decrease in the binding affinity of the metamorphosed tissues for the hormone. Further research is needed to resolve these issues.

In spite of the described differences in hormone levels, the sequence of cellular changes in experimental animals was in general agreement with observations of unperturbed metamorphic process. In both cases, larval chondrocytes were undergoing a process of cell death and autophagocytosis while perichondral cells remained healthy. The response of the larval cells to an increase in the thyroxine plasma levels was very rapid; results were observed as early as 48 h after treatment.

In this paper we have not addressed the issue of the origin of the cells that form the adult element. Circumstantial evidence from histology as well as ongoing studies involving autoradiographic labelling and removal of the larval element prior to metamorphosis indicate that the ‘stem’ cells that form the adult element are located either in the perichondrium of the larval element or in the immediately surrounding connective tissue. This is in agreement with studies on skeletal repair and endochondral ossification where it has been shown that new cell types are generated by stem cells located in the perichondrium or periosteum (e.g. see reviews by Hall, 1970 and 1978; and Albright & Misra, 1983). We believe that further exploration of this peculiar system can provide useful information on the complicated dynamics of cellular differentiation and remodelling of skeletal systems.

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