J. Anat. (1991), **176**, pp. 9–21
With 20 figures
Printed in Great Britain

Tail fin regeneration in teleosts: cell–extracellular matrix interaction in blastemal differentiation*

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(*Accepted* 1 *November* 1990)

INTRODUCTION

The tail fin of teleosts displays a skeleton composed of segmented rays that bifurcate in a proximodistal sense. Each ray shows two hemisegments, both of which form the so-called lepidotrichia. Lepidotrichia are filled with a loose connective tissue through which vessels and nerves are distributed. Distally, each lepidotrichium ends in a double palisade of rigid, fusiform collagenous fibrils called actinotrichia (Krukenberg, 1885; Marí-Beffa, Carmona & Becerra, 1989). The whole structure is covered by a non-scaled fish skin (Prenant, 1936; Becerra, Montes, Bexiga & Junqueira, 1983).

The ontogenetic development of teleost fins has been thoroughly studied (Nabrit, 1929; Géraudie, 1983). It has also been reported that, after amputation, the regenerative process is quite similar to ontogenic development (Goss & Stagg, 1957). Thus, after an initial healing of the injured part, a blastema is formed, starting from invading mesenchymal cells. The homogeneous blastemal cells proliferate and begin their differentiation towards lepidotrichium-forming cells (LFCs) in the proximal region (Marí-Beffa, Santamaría & Becerra, submitted for publication). This process lasts for some days, until the whole regeneration of the structure (Becerra, Junqueira & Montes, submitted for publication; Kemp & Park, 1970).

The formation of a blastema and the posterior regeneration of the tail fin comprise such processes as cell proliferation, synthesis of extracellular matrix (ECM) components, building of extracellular spaces, cell migration and cell differentiation. All these events are of principal importance in development (Hay, 1981; Trelstad, 1984).

Hyaluronate is the most frequent glycosaminoglycan (GAG) found in the ECM of the mesenchymal tissues during the initial stages of growth and in development (Toole, 1982). Hence, its presence has been associated with proliferative processes, and its decrease with cell differentiation (Toole *et al.* 1984). Regarding this, it has been reported that *in vitro* these molecules could participate in the control of both myogenesis and chondrogenesis (Kujawa, Pechak, Fiszman & Caplan, 1986; Kujawa & Caplan, 1986). Moreover, it has been shown that hyaluronate directly and indirectly interacts with the cell membrane by means of other GAGs, proteoglycans (PG), glycoproteins and collagen (Ruoslahti, 1989). Thus, hyaluronate seems to be an important molecule in the morphogenetic processes.

Our aim was to study the histochemical and ultrastructural characteristics of certain ECM molecules in the blastemal region of the regenerating tail fin of *Tilapia melanopleura*. Since, in this model, almost all stages of the regenerative process are

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present at different regional levels of a regenerating ray (Becerra *et al.* submitted for publication) we have focused our studies on 15 days regenerating rays. The distal region near the tip corresponds to the initial stages of the regenerative process and the proximal region to the final stages. However, the initial formation of the blastema cannot be studied in a 15 days fin and so is out of the scope of this work. The possible role played by the different extracellular matrix molecules and structures in the tip of the regenerating rays in the morphogenetic process is discussed.

MATERIALS AND METHODS

Specimens of the false mouth-breeder cichlid fish *Tilapia melanopleura*, ranging in size from 10 to 15 cm, were used in this study. The animals were maintained in glass aquaria, filled with water filtered through activated charcoal filters and with constant aeration. The photoperiod was 12 hours of light and 12 hours of darkness and the temperatures ranged from 19 to 22 °C in a climatised room. The animals were fed regularly with commercial food Sera Vipan (Sera, Heinsberg, Germany).

Partial amputations of the fins were made under anaesthesia with Tricaine (MS 222, Sigma) dissolved in water (62 mg/l) using a sharp and sterile pair of scissors. After fifteen days the regenerating tail fins were dissected out, fixed in Bouin's fluid (McManus & Mowry, 1968), embedded in paraffin and cut at 5 μm.

Histochemical procedures

The alcian blue method

This was used in order to identify the different GAGs depending on its COO^- and SO_3^- contents, according to Montes & Junqueira (1988). By adding different magnesium chloride concentrations to the alcian blue solution, different stainings of both COO^- and SO_3^- GAGs can be obtained as follows:

		Glycosaminoglycans	
Alcianophilia	$Mg\ Cl_2$ molar	COO^-	SO_3^-
+	0·2	++	—
+	0·65	++	++
+	0·9	—	++

The Picrosirius-polarisation method

This is a specific histochemical procedure for collagen detection in tissue sections. Briefly, this method consists of an initial staining of the sections in a 0·1 % solution of Sirius Red (Sirius Red F 3B 200, Mobay Chemical Corporation, NJ, USA) in saturated picric acid for 1 hour, rinsing, and eventually staining in fresh haematoxylin for 6 minutes. When the stained sections are viewed with a polarising microscope, collagenous structures can be specifically and reliably identified (Junqueira, Bignolas, Mourao & Bonetti, 1979).

Toluidine blue staining

This method, in association with enzymatic digestion, is specific for RNA.

Lectins labelled with horseradish peroxidase (HRP, Sigma)

These were used in order to reveal the presence of specific glucosidic residues in tissue sections. The following lectins were used: Concanavalin A (Con A) from *Canavalia ensiformis*, specific for mannose residues > terminal mannose > glucose > *N*-acetylglucosamine; Wheat germ agglutinin (WGA) from *Triticum vulgaris*, specific for *N*-acetylglucosamine > sialic acid (NANA). All lectins were used at a concentration of 30 μg/ml dissolved in 0·2 M Tris buffer, pH 7·6. The sections remained for 45 minutes in the lectin solution at room temperature. 3,3′diaminobenzidine was used as an electron donor to reveal the HRP activity.

Enzymatic digestion

Tissue sections were incubated in one of the following solutions, before staining in the appropriate procedure.

(*a*) *Collagenase* (Collagenase CLS IV, Worthington Biochemical Co., Freehold, NY, USA) 0·5% dissolved in 0·05 M Tris buffer, pH 7·4, containing 1 mM-$CaCl_2$, according to Montes, Becerra, Toledo & Junqueira (1982). Incubation was performed for 14 hours at 37 °C. Under these conditions, collagenase specifically degrades collagen.

(*b*) *Hyaluronidase* (testicular hyaluronidase from Sigma) 0·05% dissolved in 0·1 M phosphate buffer, pH 5·5. Incubation was carried out for 12 hours at 37 °C. Under these conditions hyaluronidase removes hyaluronate as well as chondroitin sulphates (Toole & Gross, 1971).

(*c*) *Papain* (papain IV F VIII from Difco Laboratories, Detroit, MI, USA) 0·5% dissolved in phosphate buffer, pH 4·7, containing 5 mM sodium bisulphite and 0·5 mM-EDTA, according to Junqueira *et al.* (1979). Incubation was for 24 hours at 37 °C. Papain is a non-specific protease that hydrolyses proteoglycans without affecting collagen.

In each case, adjacent tissue sections were used as controls by incubating in the corresponding buffer without enzymes.

Electron microscopy

Small fragments of regenerating rays were fixed in 2% glutaraldehyde dissolved in 0·15% M phosphate buffer at pH 7·2, followed by postfixation in 1% osmium tetroxide for one hour and overnight block staining in 0·5% aqueous uranyl acetate. Tissues were then decalcified in 5% EDTA for five days, dehydrated and embedded in a polyester resin, sectioned and double-stained with uranyl acetate and lead citrate.

RESULTS

The quick wound healing that follows the amputation is the first step in tail fin regeneration. Only three days later, the formation of a blastemal cell population occurs. These cells proliferate and begin to synthesise extracellular matrix molecules which will contribute to stabilise the structure and, not least, modulate cell differentiation, a process that starts at this time and will produce the entire restoration of the limb.

In the following description of our results, we will distinguish two different blastemal regions, namely the blastema proper (BP) and the actinotrichial blastemic region (ABR) (Fig. 1). The BP occupies a region near the tip of the regenerating ray, just under the epithelial basement membrane, and extends proximally along the centre

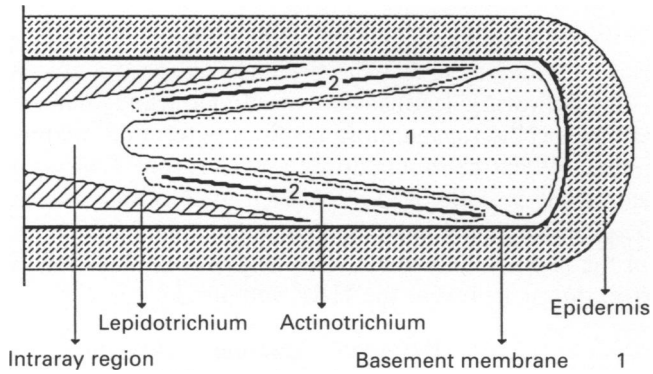
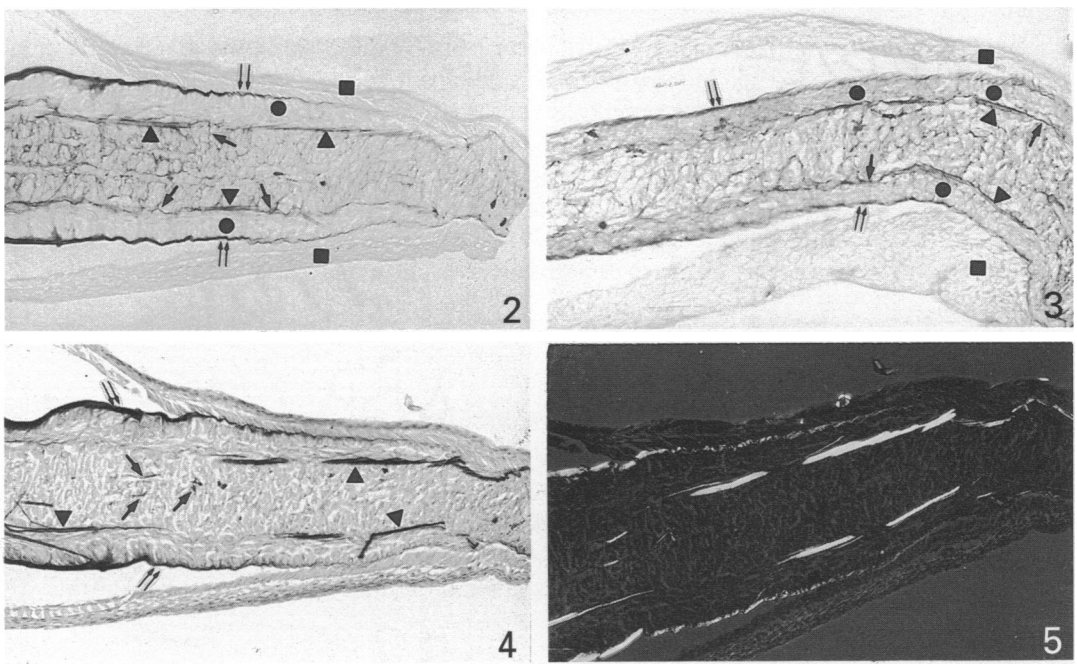


Fig. 1. Schematic drawing showing the tip of a regenerating ray. In the blastema, two regions can be distinguished according to their morphology and composition: the blastema proper (1) and the blastema around the actinotrichia or actinotrichial blastemic region (2). Other structures of the region are indicated.



Figs. 2–3. Longitudinal sections of a regenerating ray stained respectively with alcian blue solution in 0·9 M-$MgCl_2$ and 0·2 M-$MgCl_2$. Note in Fig. 2 that the central region of the blastema stains stronger proximally (left) than distally (right). In Fig. 3, on the contrary, the distal region stains more strongly. Lepidotrichia undergoing regeneration, double arrows; epidermis, squares; actinotrichia, arrowheads; network of the ABR, arrows; lepidotrichium-forming cells, circles. × 250.

Figs. 4–5. Longitudinal sections of a regenerating ray stained with picrosirius-haematoxylin and observed by light (Fig. 4) and polarisation microscopy (Fig. 5). The only structure showing birefringence (collagenous structures) are actinotrichia (arrowheads). Note the absence of collagen in the blastema; some signs of collagen appear (arrows) only in the centre of the proximal region. Lepidotrichia in formation, double arrows. × 250.

Table 1. *Staining properties of the extracellular matrix in the two regions studied, the blastema proper (BP) and the actinotrichial blastemic region (ABR) (only the extracellular network). The positive reaction is expressed with one, two or three (+). The bar separates the distal and proximal levels*

	Alcian blue*					
	0·9	0·65	0·2	PSP	Con A	WGA
BP	+	++	+++	−/++	+	+
ABR	+++	+++	+++	+	−	+++

* Alcian blue with 0·9, 0·65 and 0·2 M of $MgCl_2$. PSP, picrosirius polarisation method; Con A, concanavalin A; WGA, wheat germ agglutinin.

of the newly formed ray. ABR is located laterally some micrometres under the surface and closely related to the palisades of the actinotrichia. Moreover, the pictures have been arranged in three plates: longitudinal sections (Figs. 2–5), transverse sections stained with different histochemical procedures (Fig. 6–13), and ultrastructural features of the different regions (Figs. 13–19). Finally we propose a functional model for the regenerative process in Figure 20.

Blastema proper (BP)

In the more distal zones, the blastema displays densely packed round cells with scanty ECM among them (Fig. 14). Proximally, this feature changes; in the centre, the cells display irregular profiles and the extracellular space is prominent (Fig. 15). However in the lateral regions, near ABR, cell density and intercellular contacts increase. These peripheral blastemal cells give rise to the lepidotrichium-forming cells (LFCs) (Figs. 6, 9, 17).

The histochemical procedures used in this study clearly show that GAGs are present in the extracellular matrix in all regions of the blastema. As can be inferred from the sections stained with alcian blue at different electrolytic concentrations, sulphated GAGs predominate in the proximal regions (more mature), and hyaluronate in the distal levels (more immature) (Figs. 2, 3; Table 1). These results are confirmed in the sections incubated in papain and in hyaluronidase before alcian blue staining (Table 2).

The picrosirius-polarisation method reveals few collagen fibrils in the ECM of the distal regions of the blastema, whereas their number increases in the proximal zone, where the mature tissue of the intraray region begins to consolidate (Figs. 4, 5).

The use of both lectins (Con A and WGA) reveals a moderate positive reaction in the ECM of the region (Table 1). The staining decreases but does not disappear after papain, hyaluronidase or collagenase digestion (Table 2), thus indicating that the reaction to these lectins arises not only from the sugar residues of the GAGs and collagen but from other local glycoproteins in addition.

Electron microscopy of blastemic cells displays pale chromatin, a prominent nucleolus and discrete rough endoplasmic reticulum. The cells are densely packed and have regular borders distally, and they have irregular profiles with extracellular spaces in proximal regions (Figs. 14, 15).

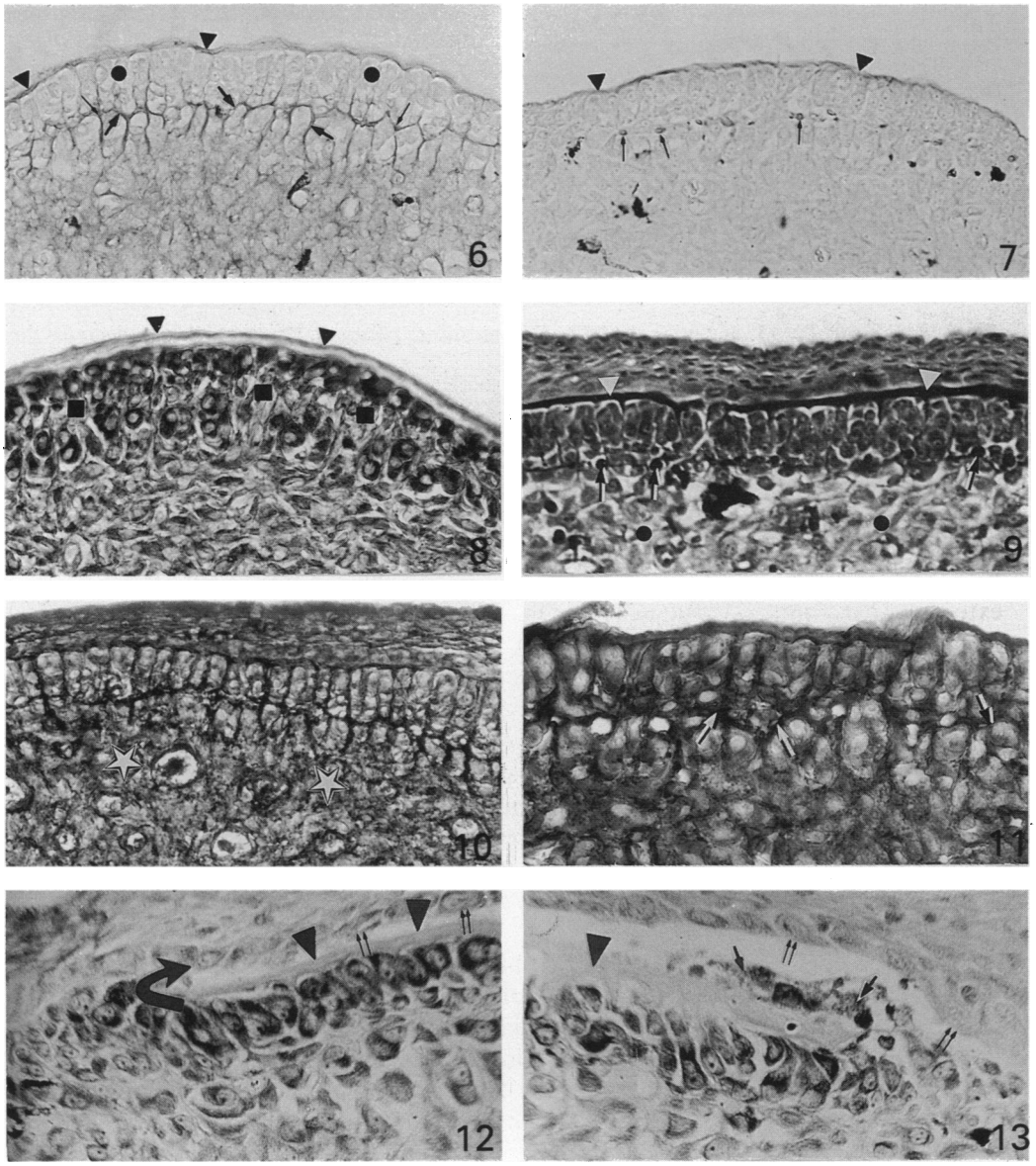


Fig. 6. Transverse section through a regenerating ray near the tip, stained with AB in 0·3 M-$MgCl_2$. The network of the ABR is strongly stained (arrows). Actinotrichia, less stained, occupy the angles of the zigzag (small arrows). Lepidotrichia-forming cells (circles) can be seen among the network and the forming lepidotrichia (arrowheads). Epidermis was detached in this section. × 350.

Fig. 7. Adjacent section to that of Fig. 6, stained with AB after digestion with papain. Note the disappearance of the network of the ABR. Papain does not seem to affect either the actinotrichia (arrows) or the nascent lepidotrichial segment (arrowheads). × 350.

Fig. 8. Transverse section through a similar level to those shown in Figs. 6 and 7, stained with Con A. Beneath the unstained lepidotrichial material (arrowheads), two bands of positively stained LFCs, form a 'sandwich', in the centre of which there is a clear band (squares). This region will be occupied by the actinotrichia and the surrounding network. × 450.

Table 2. *Staining properties of the same structures as Table I, after enzymatic digestion. 'Standard' indicates the best conditions of staining in each case (represented by + + + regardless of their intensity)*

		Alcian blue		WGA		
	Standard	P	H	P	H	C
BP	+ + +	+ +	+	+ +	+ +	+ +
ABR	+ + +	+	+ +	+	+ +	+ + +

P, papain; H, hyaluronidase; C, collagenase.

Actinotrichial blastemic region (ABR)

We consider here that actinotrichia mark an essential region in the morphogenetic process that occurs in fin fish regeneration and therefore their constituents can be clearly distinguished from the surrounding tissue. Two extracellular components can be differentiated in this region: the actinotrichia, and a filamentous network that extends from the actinotrichia to the neighbouring zone, delimiting the above-mentioned ABR (Figs. 1, 6, 10, 16, 17).

The actinotrichia are negative to alcian blue (AB), while their periphery and the network are strongly positive at all concentrations used, being highest at 0·9 M-$MgCl_2$ (Figs. 2, 3, 6; Table 1). Whereas digestion with hyaluronidase results in a slight loss of the AB stain, incubation with papain greatly decreases the reactivity to AB (Fig. 7; Table 2).

From these results it can be inferred that the extracellular network contains many GAGs, mainly sulphated, that form PGs. Hyaluronate does not appear to be a noticeable element in this location. With the picrosirius-polarisation method the network cannot be visualised (Figs. 4, 5). Consequently, collagenase digestion does not significantly modify its morphology (Fig. 11). These facts indicate that collagen is scarce at this level.

Actinotrichia and the associated framework are negative to Con A. However both blastemic cells and LFCs around actinotrichia and framework strongly bind the Con

Fig. 9. Transverse section through a level near those of the preceding figures, stained with picrosirius-haematoxylin. With this method the lepidotrichial material is completely stained (arrowheads). LFCs occupy several rows and under them, actinotrichial profiles (arrows) and some filaments of the network of ABR related with them can be observed. The intraray zone is occupied by loose connective tissue (circles). × 350.

Figs. 10–11. Transverse sections stained with WGA before (Fig. 10) and after digestion with collagenase (Fig. 11). Note the strong staining of the network. The extracellular material in the lower part, the blastema proper, is also positive (stars). Collagenase apparently does not affect the structure of the network (arrows). × 350 and × 550.

Figs. 12–13. Adjacent transverse sections through a more proximal level, stained with toluidine blue, showing patent lepidotrichial segments. Positive LFCs (curved arrow in Fig. 12) migrate along the edge of the young lepidotrichium and colonise a new space between the unstained subepidermal basement membrane (double arrows) and the unstained lepidotrichium (arrowheads). In this region LFCs (arrows in Fig. 13) show a synthetic activity towards the outer surface of the lepidotrichium. × 550.

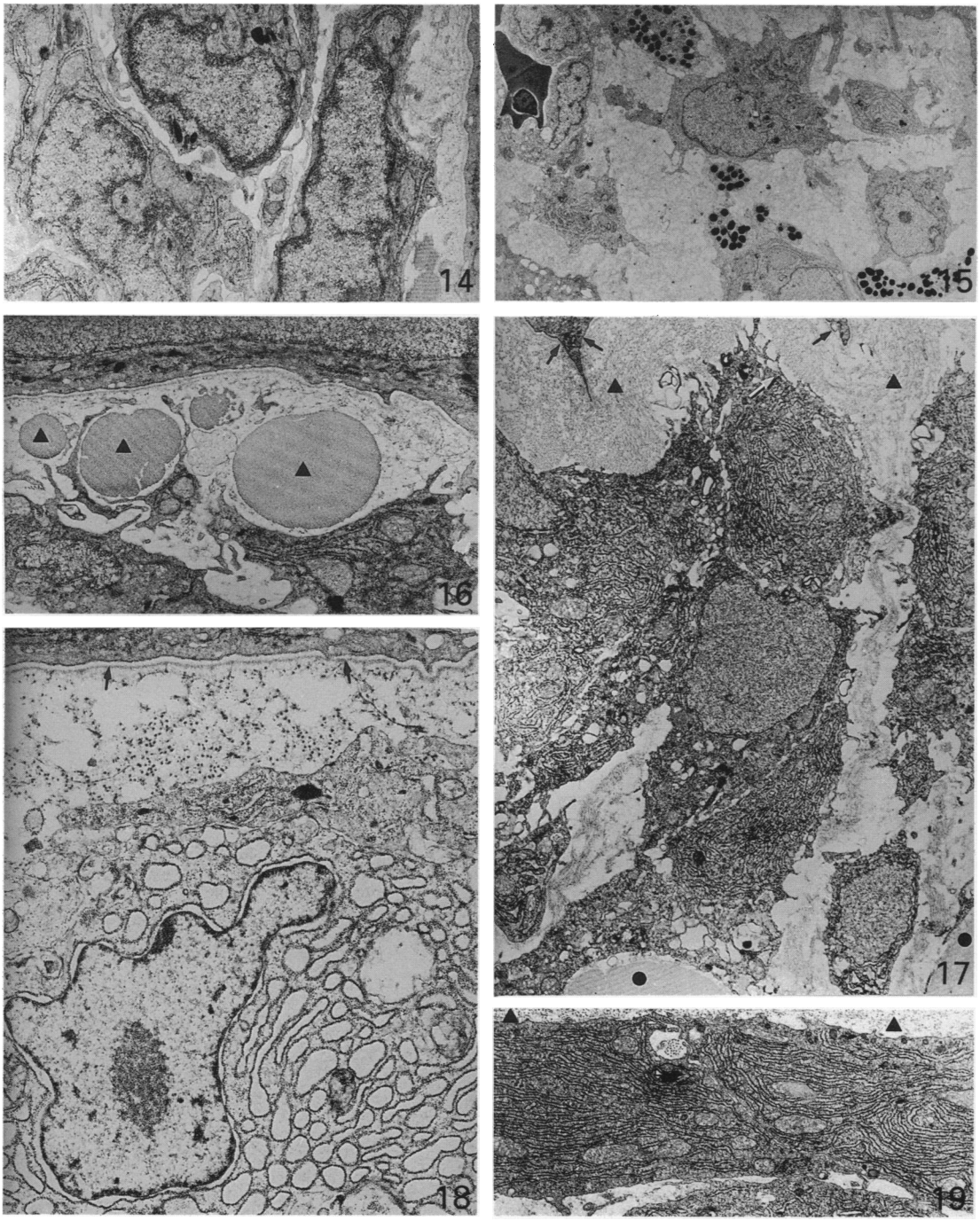


Fig. 14. Electron micrograph of cells from the distal region of the blastema. The nuclei display much pale chromatin and the cytoplasm contains rough endoplasmic reticulum cisternae. Both features indicate an important synthetic activity in these cells. The extracellular space is scanty. ×6000.

Fig. 15. General survey of cells from the proximal level of the blastema. The profiles of the cells are irregular and the extracellular space is prominent; fibrillar structures including collagen are absent. Some chromatophores and vessels are frequently found in this zone. ×4000.

A (Fig. 8). WGA lectin strongly binds to the filaments of the network (Fig. 10) and after papain, hyaluronidase or collagenase (Fig. 11) digestion the different consequences can be clearly seen (Tables 1, 2): papain destroys the positive reaction, hyaluronidase affects it rather less and collagenase does not affect it at all.

Taken together, the above results indicate that mannosyl residues (Con A negative) are absent from the network, and consequently a big family of glycoproteins can be excluded, residues of *N*-acetyl-glycosamine are present (WGA positive), presumably belonging to hyaluronate, heparan-sulphate and/or keratan sulphate. The last can be excluded since it is frequently accompanied by mannose.

Ultrastructurally the LFCs have the cytological features of typical protein synthesising cells (Figs. 17–19), having a cylindrical profile at first (Fig. 17) and becoming flattened when their activity is less (Fig. 19). Frequently the LFCs, that colonise the subepidermal space to expand the lepidotrichial hemisegment exteriorly, show the endoplasmic reticulum cisternae distended (Fig. 18).

DISCUSSION

Epimorphosis is a general mechanism by means of which the limbs of lower vertebrates regenerate until the total restoration of their shape and size. This extensive process comprises, essentially, cell proliferation and differentiation. It is known that the interaction between the cells and the ECM is, in part, responsible for the control of both processes (Hay, 1981). This interaction has been widely studied in several *in vitro* and *in vivo* models (Trelstad, 1984), but the information from teleost fin regeneration is scarce, though it has been proved that it is a good *in vivo* model for the study of the regenerative processes (Marí-Beffa *et al.* 1989; Marí-Beffa *et al.* submitted for publication).

From our results it can be concluded that, in the injured tail fin fifteen days after regeneration starts, hyaluronate is the main GAG in the distal level of the blastema, the amount of sulphated GAGs and collagen increase proximally in regions where the regenerative process is in a more advanced or mature stage. Hyaluronate could play a relevant role in the blastema, probably due to its hydrodynamic properties, by eliciting changes in the tissue hydration. Thus, an increase in hyaluronate concentration could result in a higher hydrostatic pressure acting on the cell and, consequently, in the opening of pathways for cell migration, an essential event in the morphogenetic process. This is in keeping with the studies from other authors (Zimmermann & Thies, 1984; Toole *et al.* 1984) in different experimental models.

Fig. 16. Electron micrograph of the subepidermal region of a regenerating ray. The actinotrichia (triangles) are very evident beneath the basement membrane and are surrounded by processes from the neighbouring mesenchymal cells, indicating its participation in the synthesis of the actinotrichia and/or the use of them as a substratum of migratory movements. × 8000.

Fig. 17. Electron micrograph from a level similar to that of Fig. 6. LFCs, once having crossed the palisade of actinotrichia (circles), synthesise, in a polarised fashion, new lepidotrichial material (triangles) that condenses under the subepidermal basement membrane (arrows). Note the abundance of flap parallel cisternae of rough endoplasmic reticulum. × 5500.

Fig. 18. Electron micrograph of an LFC, located between the basement membrane (arrows) and the lepidotrichium on the opposite side (not present in the picture). They contribute to the synthesis of the outer half of the lepidotrichial segment. Frequently the endoplasmic reticulum shows distended cisternae, unlike that of the inner LFCs (compare Figs. 17 and 19). × 15000.

Fig. 19. Detail of the inner LFCs close to a mature lepidotrichial segment (triangles); though LFCs are reduced in number and size, their cytoplasm is full of rough endoplasmic reticulum. × 8000.

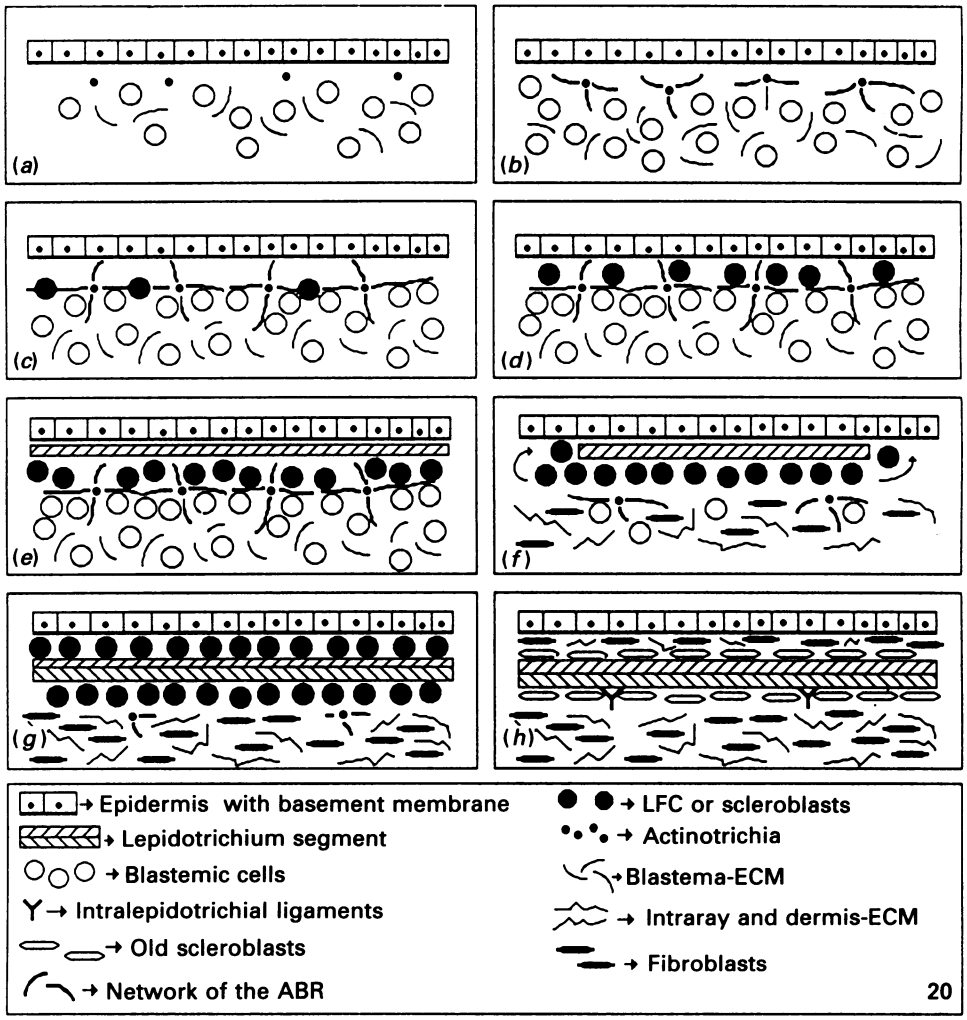


Fig. 20 (*a*–*h*). Schematic drawings representing a series of transverse sections from distal to proximal levels in a demiray in regeneration fifteen days after injury. Each picture represents a simplification of the different tissue elements.

(*a*) At the more distal region, the epidermis covers the mesenchymal cells that occupy the subepidermal space where they proliferate and release ECM molecules. Actinotrichial profiles just beneath the basement membrane soon appear.

(*b*) The blastema becomes well developed several micrometres behind. At this level hyaluronate is the main component of the ECM around the blastema cells. The palisade of the actinotrichia is well formed and a filamentous network around them appears.

(*c*–*e*) The ABR are now well developed. Some cells migrate laterally from the blastema and contact the ECM and the actinotrichia of this region. These cells then undergo changes and become LFC or scleroblasts which initiate the deposition of lepidotrichial materials in a unipolar fashion beneath the basement membrane.

(*f*) When a lepidotrichial segment is conspicuous, some scleroblasts migrate along the lepidotrichial edges; contact the basement membrane, colonise the subepidermal space and contribute to the growth of the hemisegment, releasing material at its outer face. Meanwhile the ABR begins to lessen.

(*g*) Scleroblasts in both sides of the lepidotrichial hemisegment carry on the deposition of the matrix. In this level two regions in the hemisegment can be clearly distinguished and typical fibroblasts can be found in the intraray space.

(*h*) The structure is completely consolidated. A row of flattened old scleroblasts surrounds both sides of the hemisegment. A typical loose connective tissue occupies the intraray where intralepidotrichial ligaments come from the lepidotrichial edge. Fibroblasts also appear in the dermis.

Actinotrichia are hyperpolymerised macrofibrils, composed of a characteristic collagen-like protein (elastoidin), which occupy the distal part of the rays (Krukenberg, 1885). Ontogenetically the actinotrichia are the first skeletal structures appearing underneath the fin fold (Garrault, 1936; Géraudie, 1977). However, during fin regeneration, actinotrichia appear later, after some days, though they always maintain a distal position, close to the blastema (Marí-Beffa *et al.* 1989). Besides their evident skeletal role, actinotrichia could actively participate in the morphogenetic process. It has been shown that the mesenchymal cells, invading the area and forming the early fin bud, use actinotrichia as support for their migratory movements (Wood, 1982; Wood & Thorogood, 1984); in addition, as shown in our results, the ABR related to the actinotrichia constitute a network that extends along all the blastemal region, taking shelter in a crowded cell population.

We think that the actinotrichia, together with the network related to them, form a superstructure composed basically of sulphated GAGs and, in part, of collagen. In this microenvironment the blastemal cells became stationary in their differentiation towards LFC. Therefore, the hyaluronate, present in the distal region of the blastema, can make spaces through which blastemal cells can migrate (Fig. 15). Later the cells would undergo another step in their process of differentiation, and, using actinotrichia as support, they migrate (Fig. 16) through the palisade and come into contact with the subepidermal basement membrane. This contact seems to trigger some unknown mechanism and the cells become polarised and begin the synthesis of the matrix components of the lepidotrichia and its active deposition towards the pole facing the epithelial basement membrane (Fig. 17). On the other hand, another cell population does not cross the actinotrichial boundary, perhaps due to their deep location in the blastema, and they begin to synthesise sulphated GAGs in the central region of the regenerating ray giving rise to a differentiated intraray tissue; this consists of a loose connective tissue with fibroblasts that form the intralepidotrichial ligaments and the collagenous network surrounding the vessels and nerves inside the ray (Becerra *et al.* 1983).

For some unknown reason, when some lepidotrichial material is consolidated, a new cell migration takes place. Some cells colonise the outer surface of the young lepidotrichia bordering their edge (Figs. 12, 13). Once again, the close contact between the cells and the basement membrane somehow now seems to induce them to the synthesis and the deposition of material in the outer face of the lepidotrichia. In this case the cells also became polarised but surprisingly, and contrarily to the early LFC, secretion occurs in the cell pole opposite to the epithelial basement membrane contact (Fig. 18). Thus, although both the outer and inner lepidotrichium-forming cells appear to have the same origin and function, they display differences in their morphology and polarisation.

In summary, tail fin regeneration represents a valuable model to study cell–cell and cell–ECM interaction in a morphogenetic process. These consecutive interactions would promote suitable conditions for cell differentiation.

In Figure 20 we summarise in a set of schematic drawings our current knowledge of this process.

SUMMARY

The fins of teleosts are appendices capable of regenerating by an epimorphic process that completely restores in a few days the original shape and size. After a partial amputation, the process that follows consists of: wound healing, blastema formation and cell differentiation to form a particular cell population responsible for the building of the different tissue elements.

The present work deals with the histochemical and ultrastructural characterisation of the extracellular matrix (ECM) components in different blastemal regions. In the subepidermal space of the tip of a regenerating ray, two zones can be identified: the blastema proper (BP) that occupies the more apical region and extends proximally along the centre of the regenerating ray, and the actinotrichial blastemic region (ABR), located laterally to the former, about some micrometres beneath the tip and tightly related with the palisade of the actinotrichia. Hyaluronate is the more prominent glycosaminoglycan (GAG) in BP whereas sulphated GAGs and collagen are scarce in distal portions but they can be identified proximally. In the ABR a network composed mainly of sulphated GAGs and in part of collagen is developed. This network connects the actinotrichia of each palisade and extends around them.

The significance of the ECM components in the morphogenetic processes of the regenerating rays is discussed. In addition a model for the synthesis of lepidotrichia by lepidotrichium-forming cells, differentiated from blastemal cells, is given.

This study was supported by grants from the Dirección General de Universidades e Investigación (Junta de Andalucía) and from the Comisión Interministerial de Ciencia y Tecnología (CICYT, MAR 88-0751), Spain. We are indebted to Professor Fernandez-Llebrez for his critical review of the manuscript and to Mr Cosano for typing it.

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