Regenerative capability of upper and lower jaws in the newt

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ABSTRACT The regenerating amphibian jaw represents an important model for studying pattern formation and the mechanisms underlying regeneration of facial structures. We have studied regeneration of upper and lower jaws in the urodele amphibian, Notophthalmus viridescens, using whole mount preparations stained for bone and cartilage, scanning electron microscopy and immunocytochemistry to further characterize these regenerating systems. In addition, we have investigated whether lower jaws of adults and larvae display similar regenerative ability. Although in adult animals the original shape of both the lower and upper jaws is rather faithfully reproduced following amputation, and the teeth and oral mucosa with its specialized sensory organs fully regenerate, significant differences in the regenerative ability of the various skeletal elements are observed. In fact, only tooth-bearing skeletal elements ossify, while the other elements of the regenerated skeleton remain cartilaginous for as long as 5 months after amputation. In contrast, a regenerated lower jaw in the larva is indistinguishable from an unamputated one at the same stage of development. Interestingly, regenerating adult jaws form directly bicuspids teeth, which are the type of teeth normally found in the adult, rather than the monocuspids teeth characteristic of larval jaws, indicating that jaw regeneration is not a recapitulation of development, in that an adult jaw blastema directly regenerates an adult jaw. Finally, we have studied the expression of tissue specific markers in normal and regenerating upper and lower jaws to establish whether the blastemal cells, which will form the missing part of the jaw, express any of these markers of the differentiated state, or are undifferentiated as suggested by their morphological appearance. Under our experimental conditions, no expression of markers of the differentiated state, such as those for muscle, cartilage and glands is detectable in early regenerates. On the contrary, the mesenchymal marker 22/31, whose expression in normal jaws is restricted to dermal fibroblasts and the dental pulp, is expressed in at least a half of the blastemal cells. The significance of these observations in relation to the origin of blastemal cells in the jaw will be discussed.

KEY WORDS: urodele amphibians, dedifferentiation, development, jaw, regeneration

Introduction

Urodele amphibians maintain high regenerative ability after metamorphosis, and have therefore become an important model system for studying regeneration of various body structures in adult animals (Goss, 1969; Wallace, 1981). However, most of the studies published in the last few decades have been concerned with regeneration of limbs, and to a lesser extent of tails, largely neglecting the important fact that newts also regenerate their upper and lower jaws as adults. In fact, regeneration of lower and upper jaws represents another example of epimorphic regeneration, which is defined as the reconstruction of a complex body structure via formation of a blastema, a growth zone of undifferentiated mesenchymal progenitor cells.

The original observation of this striking regenerative ability in newts comes from Spallanzani (1768), who observed that muscle, bone and teeth regrew following amputation of the jaw distal to the articulation with the paleodentary bone. At the beginning of this century, interest in jaw regeneration was revived, and a number of studies, especially on regeneration of the upper jaw and of the olfactory apparatus, were carried out (Werber, 1906; Vallette, 1929) confirming Spallanzani's observations. However, great variations in the extent of regeneration and in its time-course were observed, probably due, at least in part, to great variations in temperature at the different times of the year at which the experiments were performed. Subsequently, Goss and Stagg (1958a,b) described the major histological events occurring during regeneration of the lower jaw, and studied the regenerative jaw territory. This work showed that complete exarticulation of the mandible, either with or without the associated soft tissues, completely inhibited regeneration.

Although some information on upper jaw regeneration in the larva is available (Vallette, 1929), little is known about whether

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Abbreviations used in this paper: mAbs, monoclonal antibodies; SEM, scanning electron microscopy.
there are differences in the regenerative ability of lower jaws in adults and in the larvae. Graver (1973) reported that after amputation of a quarter or a half of the lower jaws, the dental lamina could regenerate both from anterior and posterior ends in the larva of *Ambystoma maculatum*, but only from posterior stumps in an adult *Triturus viridescens*. This work, however, was specifically concerned with regeneration of teeth in the mandible, rather than with jaw regeneration itself, and adults and larvae from two different, although related, species were used in that study.

The blastema of the regenerating newt limb is believed to originate mainly through a process of dedifferentiation of mature tissues of the stump and, in the last few years, the availability of monoclonal antibodies has made it possible to study molecular and cellular changes occurring during this process (reviewed by Ferretti and Brockes, 1991). In contrast, the origin of blastemal cells in the jaw is rather unclear, although it has been suggested that nuclei of degenerated muscle fibers accumulate at the level of amputation together with fibroblasts (Goss and Stagg, 1958a); no molecular characterization, not only of regenerating jaws, but also of unamputated ones, has been carried out to date.

From these studies it appears that the regenerating jaw can provide a valuable model for studying pattern formation and the mechanisms underlying regeneration of facial structures. It was important, therefore, to confirm and extend previous studies on jaw regeneration and to identify molecules expressed in this system in order to gain better insight both into the events leading to regeneration, and into the mechanisms of repair of facial tissues. As a first step, we have examined the time-course of jaw regeneration in whole mount preparations of adult upper and lower jaws, and in the lower jaw of stage 40 larvae of *Notophthalmus viridescens* under...
controlled environmental conditions. We have also analyzed the morphological changes occurring during regeneration of the adult jaw by scanning electron microscopy to establish whether the specialized structures of the buccal epithelium, such as glands and gustatory organs, fully regenerate. Finally, we have studied the expression of markers of the differentiated state in normal and regenerating jaws, and of a mesenchymal marker, named 22/31 (Kintner and Brockes, 1985), expressed during regeneration of the newt limb. The work presented here will provide a foundation for tackling the molecular mechanisms underlying regeneration of the jaw.

Results

Time course of regeneration of lower jaws

Adults

The functional lower jaw consists of the mandible, the hyoid apparatus and all the attached muscles which are innervated by cranial nerves. In this paper we use the terminology "lower jaw" in a more restrictive sense, since in our experiments we did not address the issue of regeneration of the hyoid apparatus. The reason for leaving the hyoid apparatus intact was to maximize the
opportunity for the operated animals to feed properly, and therefore ensure the regenerative process would proceed under optimal conditions and could be monitored over a period of months.

The mandible consists of two dermal bones, prearticular and dentary, surrounding a cartilaginous rod, Meckel's cartilage, which extends the length of the jaw (Figs. 1A, 2A, 3A-C). The two mandibles are joined at their distal ends by a median symphysis that was cartilaginous in all the normal jaws studied (Fig. 3A-B). The dentary bone, in which the mature teeth are embedded (Fig. 3A and C), covers the ventral and lateral side of Meckel's cartilage, but only a small area of the lingual side around the median symphysis. The prearticular bone, which covers the lingual side and serves for the attachment of the masticatory muscles, unlike the dentary, does not extend to the median symphysis (Fig. 1A). The more mature teeth, which are bicuspid, are arranged towards the outer margin of the bone, while the immature teeth are located in the inner border (Figs. 2A, 3A-C, 4A). Unicellular and multicellular glands are present in secretory areas of the oral mucosa lining the oral cavity, while the non-secretory areas are lined with ciliated epithelium in which non-papillary gustatory organs are embedded (see Fig. 4E-F).

We have studied the regenerative process following a transverse amputation which removed the distal fourth of the mandible and associated tissues (Fig. 1A). One week after amputation a considerable retraction of the soft tissues from the plane of amputation was observed (Fig. 2B), and a clear blastema was

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**Fig. 4.** SEM photographs of regenerating upper and lower jaws. (A) Regenerated bicuspid tooth in a 10 week upper jaw regenerate; scale bar, 50 μm. (B) Regenerated teeth and growing dental lamina (arrow) in a 10 week upper jaw regenerate. Scale bar, 50 μm. (C) Growing dental lamina (arrows) and regenerated glands (arrowhead) in a 8 weeks lower jaw regenerate. Scale bar, 200 μm. (D) Lower magnification photograph of the same jaw as in C; the arrow indicates the level of amputation, and the arrowheads regenerated glands; scale bar, 200 μm. (E) Regenerated mucous glands identical to those in the unamputated oral mucosa are found both in 10 week upper (shown here) and lower jaw regenerates; scale bar, 20 μm. (F) Sensory ciliated epithelium in a 10 week upper jaw regenerate; scale bar, 50 μm. (G) Lower jaw blastema 3 weeks after amputation (view of surface perpendicular to the mandible stump). w.e: wound epidermis; bl: blastema, scale bar, 200 μm. (H) Regenerating cartilage (c) just distal to the level of amputation (view of surface perpendicular to the mandible stump) in a 3 week lower jaw regenerate; scale bar, 200 μm.
visible 2 to 3 weeks after amputation (Figs. 3D, 4G). The blastemal cells are loosely packed in the extracellular matrix, appear to be multipolar and some of their processes seem to make contact with the overlying wound epithelium (Fig. 4G). In a 3 week regenerate it is evident, both by SEM analysis, and Alcian blue staining of whole mount preparations, that cartilage has begun to form around the inner and distal margins of the stump of the prearticular bone. This cartilaginous mass grows in a medio-distal direction and 4 weeks after amputation the two cartilaginous branches are very close at the midline (Fig. 3E), and in some animals have already fused (Fig. 2D). This growth cartilaginous rod is continuous with the existing Meckel’s cartilage and provide a scaffolding for the subsequent regeneration of the mandibular skeleton.

Six weeks after amputation the regenerating cartilage has become thicker and more elongated, the dentary bone has grown as a very thin membrane along the outermost margin of the cartilage in a proximo-distal direction (Fig. 2E), and appearance of some unerupted teeth, which parallel the growth of the the dentary bone, is observed. By 8 weeks the dentary bone has wrapped the ventral and lateral sides of the regenerated cartilage, but no bone has grown on the lingual side of the mandible, which is still completely cartilaginous. The regenerating dental lamina is clearly visible by SEM (Fig. 4C,D; see also 4B), and, although many unerupted teeth are now present, the majority of them do not have bony sockets (Table 1). By this stage glands and sensory epithelium are observed in the regenerated oral mucosa (Fig. 4D; see also 4E,F). The number of teeth in the regenerate increases in the subsequent weeks, and after 12 weeks numerous bicuspid teeth with bony sockets are observed in the regenerated dentary bone (Fig. 2F). After 20 weeks, the longest time studied so far, the mandible is more elongated, a complete row of teeth with bony sockets is observed in the regenerated dentary bone, but no regeneration of the prearticular bone is apparent (not shown). In a very few animals the two regenerated dentary bones are not separated by a cartilaginous median symphysis, but they appear to have fused at the midline, where, unlike in unamputated animals, teeth are observed (not shown).

Our observations on the major events occurring at different times after amputation are summarized in Table 1. It is important to point out that, although the sequence of events described above does not depend on the level of amputation, the time at which each event occurs changes, and a complete regenerate will form more slowly when a more proximal amputations is performed. For example, 4 weeks after amputation fusion of regenerated cartilages at the midline has occurred in jaws amputated at the more distal level (the different levels of amputation are marked in Fig. 1A), while cartilage condensation is just beginning if half of the jaw has been removed (not shown).

**Larvae**

We have studied the normal development of the lower jaw in Notophthalmus viridescens in larvae from stage 40 to stage 52, and growth of the lower jaw following amputation of its distal third (Fig. 1B). The developmental stages of Notophthalmus viridescens (Fankhauser, 1967) are not as well described as those of Pleurodeles waltl (Gallien and Durocher, 1957) and Ambystoma maculatum (Harrison, 1969), and the stages of cranial development described by Reilly (1986) in Notophthalmus do not refer to the overall stage of development. Since our observation of Notophthalmus embryos and larvae has indicated that the appearance of external features during development in this species is equivalent to that reported in Pleurodeles, but significantly different from that in Ambystoma (Ferretti and Lo, unpublished observations), we have used the
developmental tables for *Pleurodeles* as a reference for staging *Notophthalmus* embryos and larvae.

The mandible, which develops from the first branchial arch, initially appears as a small cartilaginous rod which elongates and then becomes surrounded by bone in a proximal to distal direction (see Duellman and Trueb, 1986). At the time of amputation of the jaw of stage 40-42 larvae (2-3 well formed digits in the forelimb; Figs. 1B, 5A-B), the coronary processes, which bear teeth, are present on each side of the mandible, and the prearticular bone covers about 2/3 of the mandible. The lingual side of the most distal part of the mandible is still completely cartilaginous, and many teeth, which are monocuspid, have erupted from the already formed dentary bone. Regeneration of larval lower jaws is much faster than in the adult as shown in Fig. 5C-E; 13 days after amputation the regenerating cartilages from the two mandibular stumps have always fused at the midline (Fig. 5C-D), the dentary bone has either fully (Fig. 5C) or partially (Fig. 5D) reformed, and often a complete row of erupted teeth is observed (Fig. 5C). Four weeks after amputation (Fig. 5E), when the larvae have reached stage 51-52 (3-4 digits in the hindlimb) the regenerates appear identical to the unamputated one (Fig. 5F), and in both the prearticular bone has grown distally to cover about 3/4 of the mandible (Fig. 5C and F).

**Fig. 5.** Whole mount preparations stained for cartilage (blue) and bone (red) newt larvae. (A and B) Developing lower jaws from stage 40 larvae; in (A) the lower jaw has been isolated from the skull; all amputations were performed at this developmental stage. (C-D) Jaw regenerates 13 days after amputation; some variability in the degree of regeneration is observed at this time. The regenerate in D is less developed than that in C; note that, as indicated by the arrowheads, the dentary bone and teeth have fully reformed in C, but that regeneration of the dentary bone is incomplete in D (arrowhead). (E) Jaw regenerate 28 days after amputation; note that there is no apparent difference from the unamputated control in F. (F) Unamputated control jaw fixed at the same time as the 28 day regenerate in E; by this time larvae have developed to stage 52; scale bars, 0.5 mm.

**Time course of regeneration of adult upper jaws**

The upper jaw (Figs. 1C-D, 6A) is composed of paired premaxillae that in *Notophthalmus viridescens* are fused, and paired maxillae, but here we will use the expression upper jaws to refer also to all the other tissues, soft tissues and other skeletal elements, that are removed by a transverse cut just proximal to the external naris. As shown schematically in Fig. 1C and D in all our experiments we have removed the entire dentary ramus and the pars palatina of the premaxilla, and the distal part of the dorsal ramus of the premaxilla, which forms the roof and the anterior part of the nasal capsule. The distal part (about 1/5) of the paired maxilla, which includes both part of the tooth-bearing portion and part of the wall of the nasal capsule (Fig. 7A), the distal part of the nasal capsule with the alary cartilage which is present at its anterior end, and the distal third of the vomer, were also removed. We have followed regeneration of the upper jaw up to 20 weeks (Fig. 7B), but the process appears to be completed around 15 weeks after amputation.

Because of the complex anatomical structure of upper jaws, it is difficult to produce clean cut amputated surfaces, and 2 weeks after amputation the stump edge, although perfectly healed, sometimes still looks rather jagged (Fig. 6C). A blastema is clearly recognizable in tissue sections after 3 weeks (not shown), and by 5 weeks cartilage condensation is present as a continuation of the nasal
cartilage (Fig 7C). A cartilaginous mass, clearly detectable by Alcian blue, grows at the cut surfaces of all the bones and of the nasal fenestrations over the fifth and sixth week (Fig. 6D), and by 8 weeks (6E) the regenerated cartilages appear to be well organized and are growing in a coordinated fashion. By 10 weeks, regeneration of the jaw appears rather advanced by simple visual inspection of the animal, and whole mount preparations reveal a cartilaginous scaffolding that is similar in shape to the amputated part, and in which some teeth are embedded (Fig. 6F). SEM analysis of 10 week regenerates shows the presence of bicuspid teeth proximal to the growing dental lamina (Fig. 4A-B), and of glands and sensory epithelium in the oral mucosa (Fig. 4E,F). Some individual variation in the degree of bone regeneration is observed at this time. Twelve weeks after amputation regeneration of the different bones is advanced (not shown), and in the following weeks the regenerate undergoes further growth and remodelling. By 20 weeks regeneration appears virtually complete in most animals, both in terms of reconstitution of the different tissues and of the original gross morphology (Fig. 6B). However, an important difference between the regenerated and the unamputated jaw was observed, in that the missing part of the nasal bone and of the vomer had not been replaced by bone but by cartilage.

**Cellular markers in upper and lower jaws**

We have studied the pattern of reactivity of a variety of mAbs, which had been raised against either newt or mammalian tissues, in normal and regenerating upper and lower jaws. The mAbs used and their reactivities are summarized in Table 2. The mAb LP34 reacts with many human cytokeratins and is therefore considered a pan-epithelia marker (Lane et al., 1985). In the newt, as in mammals, LP34 stained all the stratified epithelia and glands both in upper and lower jaws (Table 2). On the contrary, the mAb Kk8.60, which in mammals reacts with the keratins 10 and 11 usually expressed in cornifying epithelia (Huszar et al., 1986), selectively reacted with the buccal epithelium, but not with the epidermis (Fig 8A-B). In addition, this mAb strongly stained the dental lamina, the
outer enamel epithelium, the glands present in the buccal epithelium and under the epidermis, and, in the upper jaw, also the nasal epithelium (Fig 8A-B). In regenerating jaws LP34 stained the wound epithelium, but no Kk8.60 reactivity was observed either in the wound epithelium or in blastemal cells. An unexpected pattern of reactivity was observed in normal jaws with the mAb 116B3, that is considered a specific marker for type II collagen in higher vertebrates (Linsenmayer and Hendrix, 1980), in that not only cartilage, but also the perimysium and the gland basal lamina were strongly stained (Fig. 8C). Leu-7 (Fig. 8D), that in the newt limb is selective for Schwann cells (Gordon and Brockes, 1988), appeared to stain only nerves also in normal jaws, and the muscle marker 12/101 depicted striated muscle both in upper and lower jaws (Fig. 8E). None of these markers of the differentiated state were expressed in blastemal cells 3 weeks after amputation. On the contrary, 22/31 (Kintner and Brockes, 1985), which in normal jaws stains subepidermal cells likely to be fibroblasts and the dental pulp (Fig. 8F), was found to react with at least 50% of blastemal cells in 2 and 3 week blastemas (Fig. 8G).

Discussion

This report, not only extends previous studies on jaw regeneration, but provides novel information on the regenerative capability of different jaw tissues, on the relationship between jaw development and regeneration, and on the expression of a variety of tissue markers in normal and regenerating jaws of the newt, Notophthalmus viridescens.

Regenerative capability of facial structures

Our analysis at a gross morphological level of the major events occurring during regeneration is essentially in agreement with that by Stagg and Goss (1958a) in the lower jaw and Vallette (1929) in the upper jaw, although the times at which the various stages of regeneration occur are different. This can be explained on one hand by different levels of amputation, as indicated by Goss and Stagg (1958a,b) and confirmed by us, and by temperature variations. Temperature seemed to be poorly controlled in the experiments carried out at the beginning of the century, and even in more recent work (Stagg and Goss, 1958a,b) the operated animals were maintained at an unspecified “room temperature”, although Graver (1973) kept his regenerating animals at 16°C.

We have examined the regenerative capability of soft tissues and skeletal elements in upper and lower jaws. In both upper and lower jaws, all the soft tissues appear to have regenerated by approximately 8 to 10 weeks after amputation, and SEM analysis incontrovertibly demonstrates the regeneration of different specialized structures of the oral mucosa. Since the gustatory ciliated epithelium fully regenerates, the newt jaw may provide another valuable system for studying regeneration of sensory organs. The skeletal structure of regenerated lower jaws from different animals is very consistent, and the only significant difference observed in a few cases is ossification of the median symphysis (not shown). This may be due to the presence of some older animals in the population used in our studies, since it has been reported that the median symphysis in the salamander can indeed ossify, but quite a long time after metamorphosis (Francis, 1934). More variation among different regenerates both in the degree of ossification of maxilla and premaxilla, and in the speed at which regeneration proceeds, is observed in the upper jaw than in the lower jaw. These slight differences may be due either to individual variation, possibly related to the age of the animal, or to minor differences in the level of amputation.

Although the original shape of both lower and upper jaws is rather faithfully reproduced following amputation, some differ-
Jaw regeneration in amphibians

TABLE 2

IMMUNOREACTIVITY IN NORMAL AND REGENERATING JAWS

<table>
<thead>
<tr>
<th>mAb</th>
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<th>regenerate lower jaw</th>
<th>normal upper jaw</th>
<th>regenerate upper jaw</th>
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<td>12/101 muscle</td>
<td>striated muscle</td>
<td>—</td>
<td>striated muscle</td>
<td>—</td>
</tr>
<tr>
<td>Leu-7</td>
<td>nerve</td>
<td>—</td>
<td>nerve</td>
<td>—</td>
</tr>
<tr>
<td>II6B3 collagen II*</td>
<td>cartilage, perimysium, gland basement membrane</td>
<td>—</td>
<td>cartilage, perimysium, gland basement membrane</td>
<td>—</td>
</tr>
<tr>
<td>22/31 putative vimentin</td>
<td>dermal fibroblasts, dental pulp</td>
<td>blastemal cells</td>
<td>dermal fibroblasts, dental pulp</td>
<td>blastemal cells</td>
</tr>
<tr>
<td>Kk8.60 keratins 10, 11*</td>
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<td>—</td>
<td>nasal epithelium, lateral nasal gland, buccal epithelium, dental lamina, enamel epithelium, glands</td>
<td>—</td>
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<tr>
<td>LP34 keratins (panepith.)*</td>
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<td>wound epithelium</td>
<td>epidermis, buccal epithelium, glands</td>
<td>wound epithelium</td>
</tr>
</tbody>
</table>

*reactivity in mammals

ences in the composition of the skeletal structure are observed, and in particular it appears that only tooth-bearing skeletal elements, namely dentary, maxilla and premaxilla, can ossify, while the other elements of the regenerated skeleton remain cartilaginous for as long as 5 months after amputation. These diverse regenerative abilities are unlikely to be related to differences in the process through which the skeletal elements develop. In fact, both the prearticular bone, which is of endochondral origin, and some of the intramembranous bones of the skull are replaced by cartilage following amputation, while the dentary and the maxilla, both intramembranous bones, regenerate as bone. Since tooth-bearing bones are likely to be subjected to higher stress than other bones in the skull, a correlation between ossification of the different jaw bones and mechanical stress is conceivable. However, much more information on tensile, compressive and shearing forces in the facial skeleton (see Herring, 1993) will need to be gathered before this issue can be properly addressed in regenerating jaws.

Another skeletal defect which is consistently observed in lower jaw regenerates is that the more medial part of the dentary bone never regenerates. This observation is puzzling, since the dentary bone seems to undergo perfect regeneration both on the ventral and lateral side of the Meckel’s cartilage. One possible explanation could be that signals from the lingual side of the cartilage are different from those on the other sides, and do not support bone regeneration, either dentary or prearticular, in that location. Alternatively, growth of the dentary bone in the medio-labial location might depend on the presence of the prearticular bone, and fails to form because of the lack of this bone in the regenerate.

These results demonstrate clear differences in response to amputation between different facial structures, in that some bones fully regenerate while others appear to be replaced by cartilage, but do not exclude the possibility that bone formation might occur at much later stages. In fact, in the newt limb (Libbin et al., 1989) ossification of certain bones proceeds very slowly both during development and regeneration, although it is not understood why particular bones ossify much later than others.

Relationship to development and early events of regeneration

To compare development and regeneration within the same species, we have followed development of the lower jaw in Notophthalmus viridescens larvae from stage 40 to stage 52, and in adults and larvae following amputation of the distal third of the jaw. Although Meckel’s cartilage, bone and teeth appear in the same sequence in developing and regenerating lower jaws, regeneration of the adult jaw does not go through an intermediate stage in which the regenerate clearly presents certain characteristics of the larval jaw, such as monocuspid teeth (Miles and Poole, 1967). In fact, the teeth of the regenerating adult jaw are bicuspid as normal adult teeth, rather than monocuspid as the larval ones. This indicates that jaw regeneration is not simply a recapitulation of development, in that an adult jaw blastema directly regenerates an adult jaw. On the other hand, a larva will form a larval jaw that bears monocuspid teeth and that rapidly reaches the appropriate stage of development in relation to the rest of the body. The decision on which type of dentition will form following amputation may be under hormonal control and, since a different hormonal milieu is known to exist in larvae and adults (see Duellman and Treub, 1986), this might control morphogenesis of the teeth either directly or indirectly. At present it is not possible to determine whether larval jaws at the developmental stage 40, the stage at which the amputation is carried out, maintain some of the regulatory ability (Slack, 1980) of the undifferentiated primordium which may influence regrowth of the missing part, or whether the larval jaw undergoes epimorphic regeneration as the adult, in that the blastemal cells originate from the differentiated tissues of the stump. To gain some insight on the origin of the progenitor cells in regenerating larval jaws, we are presently comparing early regenerates from larvae and adults at the molecular level.

In regenerating jaws the origin of the blastemal cells is unclear, and it remains to be assessed whether metaplasia, defined as the ability of cells originated from a certain tissue (e.g. muscle) to give rise to a different tissue type, (e.g. cartilage), does occur. No obvious breakdown of cartilage is observed either in lower or
upper jaws of adult newts following amputation, although muscle break-down has been noticed both by Goss and Stagg (1958a) and in this study (not shown). In the lower jaw chondrogenic condensation is first observed around the inner margin of the mandible stump, suggesting that either the surrounding mesenchyme has undergone a process of dedifferentiation and metaplasia, or it contains reserve cells whose growth and differentiation is activated following amputation. Alternatively, the regenerating cartilage might represent a secondary cartilage originating from periosteal cells. This interpretation seems less likely, since Hall and Hanken (1985) have found no evidence for formation of secondary cartilage of periosteal origin during repair of fractured lower jaws in Ambystoma maculatum following injury. The fact that secondary cartilage does not seem to form in amphibians, either as a consequence of fracture or of amputation, may be related to the regenerative capability in these animals, and formation of secondary cartilage in higher vertebrates may have evolved to compensate for the lost ability of mesenchymal cells to fully dedifferentiate and revert to a progenitor cell state. Finally, it does not seem likely that the cartilaginous structure around the inner margin of the mandible stump originates as a hypertrophic response of the Meckel’s cartilage in order to mechanically compensate for the missing prearticular bone, since the onset of cartilage differentiation is not observed in continuation with Meckel’s cartilage, and no significant contribution of Meckel’s cartilage to bone repair was reported by Hall and Hanken (1985). However, in the upper jaw it is conceivable that a significant cell population contributing to the regenerate derives from dedifferentiation of the nasal cartilage, since growth of the regenerating cartilage begins around the cut edge of the nasal capsule itself.

In order to investigate whether complete dedifferentiation of stump tissues occurs during blastema formation, we have asked whether expression of certain tissue-specific markers detected in normal jaws is maintained in blastemal cells. It is important to point out, and is of interest from an evolutionary point of view, that two of the antibodies used in this study, Kk8.60 and 116B3, have a wider spectrum of reactivity in the newt (this work) than in higher vertebrates (Linsenmayer and Hendrix, 1980; Huszar et al., 1986). In mammals the mAbs Kk8.60 has been shown to be specific for keratins 10 and 11 (Huszar et al., 1986), which are considered markers of cornifying epithelia, although in non-cornifying areas of human oral epithelia patches of cells expressing keratin 10 have been observed (Morgan et al., 1987). In the newt jaw the epitope recognized by Kk8.60 is not detectable in the epidermis, but it is expressed in the buccal epithelium and in non-cornifying epithelia such as glands and nasal epithelium. The tissue distribution of keratins containing the Kk8.60 epitope might have become restricted during vertebrate evolution, as also suggested by a study
on keratin expression in a teleost fish, the rainbow trout (Markl and Franke, 1988). A similar argument can also apply to the tissue distribution of l16B3, which is specific for type II collagen in higher vertebrates (Linsenmayer and Hendrix, 1980), but that also decorates the basal lamina of muscle and glands in the newt jaw. Notwithstanding the wider tissue distribution in newt than in mammalian tissues, both Kk8.60 and l16B3 are clearly markers of the differentiated state.

The markers of different tissue types used in this study are not detectable in the blastema, with the exception of mAb 22/31, which stains dermal fibroblast and the dental pulp in normal jaws, and reacts with a high percentage of blastemal cells both in lower and upper jaws. These results show that the jaw blastema is not composed of a homogeneous cell population, and suggest that dermal fibroblasts might make a significant contribution to the blastema. It has been proposed that dermal fibroblasts play an important role in regeneration of the amphibian limb both in term of contributing cells to the blastema and in patterning of the regenerate (Slack, 1980; Tank, 1981; Gardiner et al., 1986), and it is possible that this cell type plays a fundamental role also in patterning the jaw. A thorough understanding of the origin and developmental potential of jaw progenitor cells has to await further characterization of the blastema at the cellular and molecular level, and this work is now in progress.

Materials and Methods

Animals

All experiments were carried out on either adults or larvae of the red-spotted newt, Notophthalmus viridescens (supplied either by Nasca Ltd, USA or Sullivan & Co, USA). Since it is very difficult to breed in captivity, pregnant females, which were likely to have been fertilized in the wild before collection, were used to obtain embryos. Spawning was induced by daily injection of 100 units of HCG (human chorionic gonadotropin, Sigma, Poole, UK) for 3 to 5 days. Adult news were maintained in the laboratory at 19-20°C in tanks containing slabs of floating cork, which allow the animals to climb out of the water when needed. They were fed shredded bovine heart on alternate days. Embryos were grown at 22-24°C in sterile tap water and, once at the larval stage, fed daily with brine shrimps.

Surgery

For all the surgical procedures adult animals were anesthetized in 0.1% tricaine (3-aminobenzoic acid ethylester methanesulfonate salt, Sigma, Poole, UK) and larvae were anesthetized in 1/3000 dilution in sterile tap water of a 10% tricaine solution. Amputation of lower jaws was performed by transversely cutting either the distal half or the distal third of the jaw (Fig. 1A-B), but leaving the tongue and hyoid apparatus intact. Amputation of lower jaws in larvae was performed only at the more distal level (Fig. 1A). The distal part of adult upper jaws was removed by a transverse section just proximal to the external nares (Fig. 1C-D). After surgery animals were allowed to recover from anaesthesia in a shallow aquatic solution of 0.5% sulfamerazine (Sigma, Poole, UK) for 18-24 hours before being returned to the tanks. Operated animals were maintained at 25°C for the duration of the experiment and fed as the unoperated ones. No serious problem in feeding was observed. The degree of regeneration was monitored regularly and some experimental animals were photographed immediately before and after the amputation, and at different times after surgery. The animals were sacrificed at weekly intervals after amputation, and the jaws collected for further analysis.

Analysis of regenerating jaws

Regeneration of cartilages and bones was studied in whole mount preparations stained for these tissues with alcian blue and alizarin red by minor modifications of Simons and Van Horn's method (1971).

For histological studies jaw blastemas were removed at different times after amputation and fixed overnight in 4% paraformaldehyde in 100 mM phosphate buffer, 120 mM NaCl, pH 7.4 (A-PBS). The jaws were decalcified by treatment with 0.5 M EDTA, pH 7.5, for 3 to 5 days. After rinsing in the same buffer the blastemas were embedded in paraffin, and 6-8 μm sections cut. Sections were then stained either with alcian blue and Durazol red or with Harris' haematoxylin and eosin.

Scanning electron microscopy was performed in tissues fixed overnight in 4% paraformaldehyde in PBS. The fixed tissues were dehydrated in ethanol, transferred to acetone and then critical point dried using carbon dioxide. Eight and 10 weeks regenerates were analyzed intact, while 3 weeks lower jaw regenerates were cut perpendicular to the mandible stump into six blocks in order to visualize both the blastemal cells and changes in the regenerate at different distances from the amputation plane. The specimens were sputter coated with gold/palladium and visualized at 15 kv in a Cambridge 90B stereoscan scanning electron microscope.

Immunohistochemistry

Reactivity of the monoclonal antibodies (mAbs) Kk8.60 (Huszar et al., 1986), LP34 (Lane et al., 1985), 22/31 (Kintner and Brockes, 1985), Leu-7 (Gordon and Brockes, 1988) and l16B3 (Linsenmayer and Hendrix, 1980) was assayed on 8 μm cryostat sections of unfixed normal jaws and sections of 2, 3 and 4 week regeneration blastemas. The sections to be stained with 22/31, Kk8.6, LP34 were always fixed with cold acid-alcohol (95% ethanol-5% acetic acid, Ferretti et al., 1989). The sections for 12/101 and Leu-7 staining were fixed either with acid-alcohol or with 4% paraformaldehyde (PFA) in A-PBS for 5 minutes at room temperature. For l16B3 staining PFA fixed sections were digested for 1 hour at room temperature with 1 mg/ml testicular hyaluronidase (Type IV-S, Sigma) and then processed as previously described (Fitch et al., 1989). Bound antibodies were detected by a rhodamine-conjugated-rabbit anti-mouse-immunoglobulin antibody (Dako, Denmark) and the nuclei stained with 1.25 μg/ml of Hoechst dye 33258 (Sigma).

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References


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