Radiologic Study of Limb Regeneration in the Axolotl

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For years the study of regeneration involved the sacrifice and histological preparation of the animals at different stages. Our study was undertaken to assess the feasibility of using radiography as a tool for the continuous study of the limb regeneration without the need to sacrifice the animals. Sixteen axolotls (*Ambystoma mexicanum*) each received two amputations, a forelimb and a contralateral hindlimb, one at the zeugopodial (distal) level and the other at the stylopodial (proximal) level. The animals were radiographed at intervals over the next 273 days. This technique showed clearly the sequence of ossification in the regenerative limb as well as the development of a number of skeletal abnormalities, particularly in the autopodial elements. There also appear to be a number of similarities between embryological development and regeneration in that they both share a proximo-distal chronological pattern of ossification and that with each segment the bones ossify in a similar manner.

Since the turn of the century, a great deal of effort has been spent in the study of limb regeneration. This process occurs most readily in the urodele amphibians, and of this group the axolotl (*Ambystoma mexicanum*) has been the animal most often studied. One problem with past studies has been that regeneration, instead of being looked upon as a dynamic process, has simply been studied via series of histological sections. Researchers would allow the animals to regenerate for a certain time, at which point they were sacrificed and their limbs examined by means of histological preparations. Although this provided a reasonably complete overview of the morphological and cellular changes occurring within the early blastema, most studies limited themselves to this time period and ignored most of the period of osteoregeneration, which occurs much later in the regeneration process. The aim of this study was to assess the feasibility of using radiography, particularly soft x-rays, to study the progression of skeletal regeneration. In this way it can be followed in its entirety without the need to sacrifice the animal.

Materials and Methods

Sixteen axolotls (*Ambystoma mexicanum*) were obtained from the breeding colony of Dr. R. Gordon. They were kept in tanks containing a 1: 1 mixture of distilled and tap water maintained at 18°C ± 2° and fed every two days on raw beef heart. When all of the animals had reached an overall length of at least 6 cm, they were anaesthetized using a solution of 0.03% Tricaine Methanesulfonate (MS-222) buffered to a pH of 7.0 (Scadding, 1989) and were then radiographed by placing them directly on film (in a visually opaque envelope) with the limbs pressed flat against the surface. The radiography was done in an enclosed stationary anode unit (Faxitron). The exposure was one minute at 20 kV, which gave a total body dosage of 31 mR as measured using an integrating dosimeter (Victoreen 471RF). The film used was Kodak X-Omat TL, which was wrapped in plastic in order to keep the paper envelope dry; it was developed in a Kodak RP X-omat 90 second automatic processor. Following the exposure, each animal had two limbs amputated: a hind limb and the contralateral forelimb, one at the zeugopodial level and the other at the stylopodial level. The axolotls were then radiographed at intervals over the next 273 days, until the size of the regenerate was equal to the size of the opposite unamputated limb. During this period each animal received 18 x-rays with a cumulative full body dose of 558 mR. During the course of the study two axolotls died from Costia (protozoan) infections.

Results

Day 4. This was the first post-amputation film. Because of the very low kilovoltage used, the soft tissue could be seen well. In all of the animals, there was a thin epidermal layer covering the amputation site. This corresponds with the wound healing stage as described by Tank et al. (McLaughlin et al., 1982)

Days 6, 8 and, 12. An increase in the amount of soft tissue distal to the bone end was demonstrated, and the distal stump was beginning to take on a more conical shape. Some evidence of osteoclastic resorption of the distal bone ends could be seen in a few animals by Day 8 and in all by Day 12. By then a radiolucent band had also appeared just proximal to the bone end. Comparison with later films showed that this delineated the full extent of the bone resorption.

Days 16 and 20. Bone resorption had progressed, but it ceased by Day 20. Distal ,soft tissue length had increased, and the apical epidermal lobe could now be seen. In those animals whose amputations were just distal to the elbow joint, the radial and ulnar remnants were completely resorbed.

Days 24, 28 and 32. There were no bony changes during this period. On careful examination of the forelimb soft tissue on the Day 24 film, the first distal cleft could be distinguished. By Day 32 the first cleft had become better developed, and the second and third clefts had appeared. Development of the first cleft in the hindlimb area was not seen until the 32 Day film.

Day 40. The first evidence of calcification was seen in the forelimbs, just distal to the amputation sites; none was yet visible in the hindlimb area.

Day 47. Calcification could now also be seen in the radius and ulna in the forelimbs with midhumeral amputations, but none was visible in the autopodia. In all cases, calcification occurred in a proximo-distal direction. The hindlimb now had the same degree of calcification as the 40 Day forelimb.

Day 57. Calcification was seen in all of the forelimb but only some of the hindlimb autopodial metacarpals and phalanges. There was an increase in the amount of digital separation and the bone density appeared to have increased.

Day 78. The length and density of all skeletal elements had increased, and all the autopodial tubular bones in both the forelimbs and the hindlimbs were now well ossified. At this point a number of skeletal anomalies become evident. There were also three humeral fractures, which may have been a result of handling.

Days 92 and 100. The site of amputation was still visible in all cases, seen as a density interface between the original bone and the incompletely mineralized regenerating bone.

Day 178. The regenerated limbs were now only slightly smaller than the normal limbs. Cortical bone density had increased significantly, but was still much less than the original bone. Very little cancellous bone was seen within the diaphyses.

Day 273. The length of the regenerated limbs was approximately equal to the unamputated limbs, and the junction between the new and old bone was barely visible due to an increase in the amounts of cortical and cancellous bone. The axolotls had undergone a rather large increase in overall size since Day 178.

A number of abnormalities were noted in the regenerated limbs, and these were distributed almost equally between forelimbs and hindlimbs. Of the 12 assessable stylopodial amputations, 9 (75%) demonstrated defects, and of the 14 assessable zeugopodial amputations, 8 (57%) showed defects. The types of abnormalities seen are listed in Table 1, with the most striking being single examples of radioulnar fusion, metatarsal fusion, and longitudinal deficiency of the tibia. The abnormalities occurred most often in the autopodial elements.

Discussion

Previous studies of axolotl and amphibian limb regeneration have used histological techniques, sacrificing animals at specific stages and concentrating mainly on the blastema and possible chemical mediators. To our knowledge, only one other study has made use of diagnostic x-ray techniques for studying regeneration (Brunst, 1950). The authors measured the amount of bone regression after amputation and undertook soft tissue measurements to describe growth characteristics.

In this study our aim was to use radiographs to follow the continuous progression of regeneration within individual animals without the need to sacrifice them. This technique proved particularly effective in demonstrating the development of calcification and ossification. For more detailed studies of exact ossification sequences, the use of larger animals and more frequent x-rays would be of value.

When comparing our results to the regenerative phases seen in other studies, there appears to be good correlation. Most of the gross morphological aspects of Tank's classifi-

Table 1

Abnomality	Number
Polydactyly	5
Missing phalanges or failure of phalangeal ossification	5
Complete autopodial or ray hypoplasia	5
Polyphalangeal digits	3
Miscellaneous	3

cation system (McLaughlin et al., 1982) can be seen, and some of the cellular changes, such as increasing osteoclast numbers can be inferred from our films. In addition, the changes in bone density we observed between Days 90 and 170, and between Days 170 and 273 correlate well with work done by Libbin et al (Libbin et al., 1989). Using atomic absorption spectrophotometry, their study showed that between 90 and 180 days, calcium content of the newt regenerate doubles, reaching 25% of the normal value, and that by 270 days the calcium content was 75% of control value.

Several recent studies have suggested that limb regeneration mirrors embryological development, including work on newt carpal ossification patterns (Tank et al., 1977), scanning electron microscopy of the axolotl limb bud and blastema (Smith et al., 1974), and work on limb patterning (Stocum, 1975; Puckett, 1936). Our findings also show similarities between the two processes in that ossification chronologically proceeded in a proximo-distal pattern, with forelimb preceding hindlimb, and that the ossification between Days 47 and 273, took place in much the same way as the three developmental phases of the newt described by Mather and Goel (Libbin et al., 1988): "(i) peripheral calcification in the middiaphyseal region; (ii) peripheral calcification in the entire diaphysis accompanied with central calcification in the middiaphyseal region; and (iii) peripheral and central calcification of the entire diaphysis...." If limb regeneration does in fact replicate embryological development, then limb regeneration may provide a model for studying the pathogenesis of congenital abnormalities of the limb.

The developmental abnormalities seen in the regenerated limbs in our series (Table 1), were similar to those seen in other series (Hinchliffe and Johnson, 1980), and the bony evolution of these abnormalities was clearly shown by our technique. One shortcoming of this study though was the inability to visualize the mesenchymal and cartilaginous phases of the regenerating bone. This is crucial when examining defects such as syndactyly and suppression of an element, because both are thought to originate during this time: syndactyly via coalescence of mesenchyme or secondary fusion of cartilaginous elements and suppression via absence of mesenchymal condensation, condensation with regression, or cartilaginous regression (Foret, 1973). Magnetic resonance imaging (MRI), which shows soft tissues and cartilage well, might be of particular value in assessing these early stages.

In one reported study, autopodial abnormalities occurred more frequently following distal as compared to proximal amputations (Hinchliffe and Johnson, 1980), whereas in our animals there was a 75% defect rate with proximal (stylopodial) amputations and a 57% rate with distal (zeugopodial) amputation. However, ours was a relatively small series, and the effects of ionizing radiation and the recurrent administration of anesthetic are possible confounding factors. Studies on the effects of radiation on the regenerating limb have been done, but these have looked at the single dose necessary to halt the regeneration process altogether and not at the induction of abnormalities (Robinson and Scadding, 1983; Mathur and Goel, 1976). Because our dose is fractionated and is approximately 530 times smaller than the dose needed to suppress regeneration, it is unlikely that x-ray dose would have a major effect. The anesthetic on the other hand may have an inductive effect because of its known tendency to increase cAMP levels within the regenerating limb (Tank et al., 1976; Muneoka and Bryant, 1982), but caution was taken to use the lowest effective concentration possible (Scadding, 1989).

This study has shown that radiography is a useful method for studying limb regeneration. It may also, therefore, have potential for increasing our understanding of normal and abnormal limb development.

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