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Development and regeneration of the neonatal digit tip in mice

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Abstract

The digit tips of children and rodents are known to regenerate following amputation. The skeletal structure that regenerates is the distal region of the terminal phalangeal bone that is associated with the nail organ. The terminal phalanx forms late in gestation by endochondral ossification and continues to elongate until sexual maturity (8 weeks of age). Postnatal elongation at its distal end occurs by appositional ossification, i.e. direct ossification on the surface of the terminal phalanx, whereas proximal elongation results from an endochondral growth plate. Amputation through the middle of the terminal phalanx regenerates whereas regenerative failure is observed following amputation to remove the distal 2/3 of the bone. Regeneration is characterized by the formation of a blastema of proliferating cells that appear undifferentiated and express *Bmp4*. Using chondrogenic and osteogenic markers we show that redifferentiation does not occur by endochondral ossification but by the direct ossification of blastema cells that form the rudiment of the digit tip. Once formed the rudiment elongates by appositional ossification in parallel with unamputated control digits. Regenerated digits are consistently shorter than unamputated control digits. Finally, we present a case study of a child who suffered an amputation injury at a proximal level of the terminal phalanx, but failed to regenerate despite conservative treatment and the presence of the nail organ. These clinical and experimental findings expand on previously published observations and initiate a molecular assessment of a mammalian regeneration model.

Keywords: Regeneration; Mammal; Digit; Finger; Blastema; Ossification

Introduction

Fingertip regeneration in children has been reported in the clinical literature when amputation injuries are conservatively managed and allowed to heal by secondary intention (Stevenson, 1992). Conservatively managed amputation injuries restore the digit contour, the fingerprint, normal sensibility and digit function and heal with minimal scarring. Lengthening of the finger is described in some cases; however, it is not always clear whether finger elongation results from regeneration of the terminal phalangeal bone and/or by distal growth of granulation tissue (Douglas, 1972; Vidal and Dickson, 1993). Observations on the healing of fingertip amputation injuries make it difficult to distinguish between a wound healing response that gives excellent cosmetic and functional repair of soft tissues, from a regeneration response that, in addition, completely or partially restores skeletal structure. Regeneration responses in lower vertebrates, such as the salamander, involve complete skeletal replacement (Bryant et al., 2002), thus whether or not there is skeletal regrowth following fingertip amputation is key to establishing whether a regeneration response is stimulated. We are aware of a limited number of documented cases of bone regrowth following an amputation injury in humans (Lee et al., 1995; Vidal and Dickson, 1993). Based on the clinical literature we can conclude that the human fingertip possesses some regenerative capacity; however there is insufficient documentation to predict whether or not a regenerative response will occur for any given amputation injury.

Digit tip regeneration in rodents has been used as an experimental model to study regenerative mechanisms in mammals, in particular, as it relates to fingertip regeneration

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in children. In both neonate and adult rodents, digit tip regeneration involves wound healing with regrowth of the terminal phalangeal bone (Neufeld and Zhao, 1995). The regeneration response is level specific and restricted to amputation through the terminal phalangeal bone (Borgens, 1982; Neufeld and Zhao, 1995; Reginelli et al., 1995). Regeneration in both children and rodents is noted to be associated with the presence of the nail organ at the wound site, and ablation/transplantation studies in rodents suggest that the nail organ plays a crucial inductive role in the response (Mohammad et al., 1999; Neufeld and Zhao, 1995). During digit development in mice and humans, the nail organ forms in association with distal mesenchymal cells that prominently express transcripts for *Msx1* and *Bmp4*, and these genes have been shown to be functionally required for the embryonic regeneration response (Allan et al., 2006; Han et al., 2003; Reginelli et al., 1995). Thus, the evidence implicates either the nail organ itself and/or cells associated with the nail organ as playing a critical role in digit tip regeneration.

In higher vertebrates, the terminal phalangeal bone is the only bone known to have the capacity to fully regenerate following an amputation injury. Like other long bones of the limb, the terminal phalanx initially forms by endochondral ossification, first forming a chondrogenic template that is later replaced by bone. Unlike classically studied long bones, the terminal phalanx possesses only a single growth plate located at its proximal end, so it is best described as equivalent to the proximal half of a typical long bone (Dixey, 1880–1881). Typical long bones increase in length by endochondral growth occurring at the epiphyseal plates and involve cartilaginous growth with bone replacement. In contrast, long bones increase in diameter by appositional ossification that occurs along the diaphysis and involves the progressive laying down of new bone directly onto the surface of existing bone, a process that does not involve cartilage formation. In the case of the terminal phalanx, bone elongation appears to occur by endochondral growth proximally and appositional ossification distally.

Since the terminal phalanx is the only bone of the mammalian limb that has retained regenerative ability, we have carried out studies to characterizing its formation, elongation and regeneration in the neonatal mouse. We developed the postnatal day 3 (PN3) digit tip as a model for regeneration and we show that amputation through the distal region of the terminal phalanx results in a regeneration response whereas amputation through the proximal region does not. We find that when neonatal regeneration occurs it is not perfect; the regenerated bone is anatomically correct but never attains the length of unamputated digits. Our studies provide evidence that neonatal digit tip regeneration involves the formation of a blastema of proliferating cells expressing developmentally relevant genes, and the differentiation of bone tissue by direct ossification. Finally, to provide a link between experimental and clinical findings, we present a case report of a proximal amputation injury to the fingertip of a 28 month old child that was conservatively treated and resulted in a wound healing response without evidence of bone regrowth. This case report identifies a fingertip amputation level in children, similar to a proximal amputation in neonatal mice, which is unable to mount a regeneration response.

Materials and methods

Digit tip amputation and tissue collection

All mice used in this study were outbred CD#1 strain supplied by Charles River Laboratories (Wilmington, MA). Following anesthetizing with an intraperitoneal injection of Nembutal (Pentobarbital; 50 mg per kg body weight) or Ketamine/Xylazine (Ketamine 80 mg and Xylazine 8 mg per kg body weight), we utilized the central three digits (digits 2, 3, 4) of the hindlimb of postnatal day 3 (PN3) neonates for digit formation and regeneration studies. Amputations were carried out using microdissection scissors. For distal amputations the terminal phalangeal bone was transected at its midpoint so that 50% of the bone remained in the stump tissue. For proximal amputations the transaction level removed between 67% and 75% of the terminal phalangeal bone leaving 25–33% of the bone in the stump tissue. At PN3 the distal amputation level transects forming bone tissue while proximal amputations transect cartilage (Figs. 2A and B). For all of our studies we treated the central three digits of the hindlimb as equivalent. For histological and in situ hybridization analyses digit tissues were obtained from mice at embryonic day 17.5 (E17.5), E18.5 (birth), PN3, PN7, PN14 and PN21. Procedures for care and use of mice for this study were in compliance with standard operating procedures (SOPs) approved by the Institutional Animal Care & Use Committee (IACUC) of Tulane University Health Science Center or the University of California Irvine.

Histological analysis

Differential whole-mount bone staining of developing or regenerated digits was performed essentially as described by McLeod (1980) with minor modification. Briefly, digits of neonates (younger than 3 weeks) were fixed with 100% EtOH, skinned, delipidated in acetone and stained with Alcian Blue 8XG/Alizarin Red S in 5% acetic acid, 95% EtOH. Stained tissues were treated in 1% KOH and cleared by glycerol. Mice older than 3 weeks were stained simply with Alizarin Red S because cartilage tissue is absent in the digit tips. Adult digit samples were stained with 0.002% Alizarin Red S solution in 2% KOH. Samples were washed with 2% KOH and Mall's solution and stored in glycerol. For paraffin sectioning tissues were fixed in 4% paraformaldehyde, dekeratinized in 1% KOH, decalcified in Decalcifier II (Surgipath, Richmond, IL) and stained with Mallory's triple stain (Humason, 1962).

Bone length and ossification

To assess growth rate we directly measured the proximo-distal length of the terminal phalanx in whole-mount preparations ($n=8$ per time point). Postnatal ossification was determined by analyzing calcein labeled whole-mount samples. To determine new bone formation, calcein was injected intraperitoneal (10 mg/kg body weight) and digit tissue analyzed 1 day later using fluorescence microscopy (Suzuki and Mathews, 1966). In other experiments calcein was used as a vital marker to label existing bone and non-labeled bone distal to the calcein label was measured to estimate subsequent ossification. In some cases we used a double labeling technique in which new bone was labeled with Alizarin Red (Neufeld and Mohammad, 2000).

In situ hybridization

Section in situ hybridization was performed to characterize gene expression during digit tip development and regeneration as previously described (Han et al., 2003). In situ probes for *type II collagen* (Kosher et al., 1986; Nah et al., 1988), *Indian hedgehog* (Bitgood and McMahon, 1995), *type X collagen* (Schmid and Linsenmayer, 1985), *type I collagen* (Kosher et al., 1986) and *osteocalcin* (Komori et al., 1997) were used to characterize differentiation of skeletal elements. In situ probes for *Msx1*, *Msx2* and *Bmp4* were used to characterize the expression of developmental genes during regeneration (Han et al., 2003).