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Neurotrophic regulation of fibroblast dedifferentiation during limb skeletal regeneration in the axolotl (*Ambystoma mexicanum*)

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ABSTRACT

The ability of animals to repair tissue damage is widespread and impressive. Among tissues, the repair and remodeling of bone occurs during growth and in response to injury; however, loss of bone above a threshold amount is not regenerated, resulting in a "critical-size defect" (CSD). The development of therapies to replace or regenerate a CSD is a major focus of research in regenerative medicine and tissue engineering. Adult urodeles (salamanders) are unique in their ability to regenerate complex tissues perfectly, yet like mammals do not regenerate a CSD. We report on an experimental model for the regeneration of a CSD in the axolotl (the Excisional Regeneration Model) that allows for the identification of signals to induce fibroblast dedifferentiation and skeletal regeneration. This regenerative response is mediated in part by BMP signaling, as is the case in mammals; however, a complete regenerative response requires the induction of a population of undifferentiated, regeneration-competent cells. These cells can be induced by signaling from limb amputation to generate blastema cells that can be grafted to the wound, as well as by signaling from a nerve and a wound epithelium to induce blastema cells from fibroblasts within the wound environment.

Introduction

The ability of animals to repair tissue damage in response to injury is widespread and impressive. In some instances, this ability extends to regenerating most of the entire body (e.g. planaria); however, for most vertebrates, including humans, regeneration is restricted to the level of tissues such as bone, muscle, nerves and blood vessels. In spite of the ability to regenerate individual tissues, regeneration of organs that integrate the structures and functions of the individual tissues does not occur. A notable exception is urodele amphibians (salamanders), which are unique among adult vertebrates in their ability to regenerate complex organs, including limbs. Among the tissues and organs that can regenerate in salamanders, the limb has been most extensively studied, allowing for much of our current understanding of the mechanisms regulating organ regeneration in adult tetrapods. The challenge of regenerative medicine is to discover how to integrate and orchestrate the regenerative responses of the various component tissues in order to regenerate functionally and structurally complex organs (Bryant et al., 2002; Endo et al., 2004).

The skeleton is one of the tissues that can regenerate in humans and other tetrapods. Bone and cartilage are dynamic tissues that are constantly being remodeled during growth in youth, as well as in old age. In addition, injuries to the skeleton typically heal well and small structural deficiencies are replaced. The endogenous regenerative response of mammalian bones is well understood and initially involves formation of a soft callus (fibrocartilage) derived from mesenchymal progenitors (Schindeler et al., 2008). This structure provides mechanical support to the fracture, and is the template for the eventual formation of woven bone that restores structure strength to the regenerated bone. This intrinsic regenerative response can heal the end of apposed bones as well as fill in small gaps between the ends of the broken bones. In the later case, this gap can be progressively widened with progressive callus formation, which is the basis of distraction osteogenesis therapies to elongate skeletal elements.

In spite of this intrinsic regenerative ability, loss of bone above a threshold level, a "critical-size defect" (CSD) is not regenerated (Schmitz and Hollinger, 1986). The challenge to regenerate a CSD is a major focus of therapies in regenerative medicine. Most of these therapies are designed to orchestrate the interaction of grafted cells with the potential to regenerate the skeleton, with scaffolds that bridge the CSD. In the end the goal is to provide a new skeletal element that integrates into and has the structural properties of the endogenous skeleton (Schmidmaier et al., 2008). In spite of progress in achieving these goals, long bone defects remain an unsolved and challenging clinical problem.

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As in humans and other vertebrates, salamanders also fail to regenerate a CSD, in spite of their ability to regenerate an entire amputated limb (Goss, 1969; Hutchison et al., 2007). A salamander limb is composed of the same tissues as a human limb, and each regenerates by a comparable mechanism involving the growth from existing tissues in the stump (nerves and blood vessels) or from adult stem cells, such as muscle satellite cells (Gardiner, 2005; Kragl et al., 2009; Morrison et al., 2006). Nevertheless, as in mammals, a mid-diaphyseal radial deletion is not regenerated (Hutchison et al., 2007). Unlike mammals, the salamander arm does have the potential to regenerate the missing skeletal element. If the ulna is removed surgically and the limb is amputated, the ulna is regenerated distal to the amputation plane even though it was absent in the proximal stump at the time of amputation (Goss, 1969). Of relevance to our study is the observation that although the distal ulna is regenerated, the proximal defect is not (Goss, 1969). Therefore the limb stump cells have the ability to regenerate an excised ulna (equivalent to a CSD), and do so in response to signals that induce dedifferentiation and blastema formation (Bryant et al., 2002), but fail to do so in the absence of those signals.

These classic studies (Goss, 1969) are a reminder that a lack of regeneration (such as a CSD) is not necessarily a consequence of a lack of regenerative ability, but may result from a failure to progress through the early, requisite steps of regeneration (Muller et al., 1999). Collectively these early steps result in blastema formation via a developmental process referred to as "dedifferentiation." In spite of the essential role of dedifferentiation in salamander limb regeneration, little is known about this phenomenon at the level of cellular and molecular mechanisms. The current operational definition of dedifferentiation is that it is the process by which a blastema is formed from progenitor cells present in the uninjured limb, and involves the reacquisition of embryonic-like developmental potential as indicated by the re-expression of embryonic genes (Han et al., 2005; Satoh et al., 2008b). By this view, cells in the limb stump revert to an embryonic state (limb bud), and regeneration progress as a recapitulation of limb development. Thus, dedifferentiation is the early phase of regeneration leading to formation of blastema cells that function like limb bud cells to re-develop the lost limb structures.

Although progenitor cells for all the limb tissues eventually contribute to the blastema, the early blastema is derived from cells within the connective tissues of the injured limb (Bryant et al., 2002; Kragl et al., 2009; Muneoka et al., 1986). Cells from other tissues are lineage restricted (e.g. myoprogenitor cells derived from muscle-associated satellite cells) and migrate into the blastema after it forms and begins to grow (Bryant et al., 2002; Kragl et al., 2009). Cells from the connective tissue (fibroblasts) reacquire the developmental potential their progenitors had in the embryo, and give rise to the connective tissue-related tissues of the regenerated limb; including cartilage, bone, ligaments, tendons and loose connective tissue (Kragl et al., 2009; Lheureux, 1983; Muneoka et al., 1986). In addition, these cells within the connective tissue of the limb are the source of the signals that regulate growth and pattern formation during regeneration (Bryant et al., 2002; Endo et al., 2004; Gardiner et al., 1986; Muneoka and Bryant, 1984; Rollman-Dinsmore and Bryant, 1982). Therefore, the challenge to inducing regeneration of skeletal defects lies in understanding the mechanisms regulating the state of differentiation and developmental potential of connective tissue fibroblasts.

The phenomenology of fibroblast dedifferentiation and blastema formation are well understood, even though the molecular details of the underlying signaling pathways are not. The importance of a specialized regeneration epithelium has long been recognized (Tassava and Garling, 1979; Thornton, 1957; Thornton, 1960), and its formation and function is induced and dependent upon signaling from the nerve (Satoh et al., 2008b). Nerve signaling appears to be required at additional steps in the regeneration cascade. The loss of nerve signaling (denervation) or the loss of a functional regeneration epithelium (e.g. surgical removal or the grafting of mature skin to inhibit its formation) results in the same phenotype, regenerative failure. Although most studies have focused on dermal fibroblasts as the target cells of nerve/RE signaling, fibroblasts in the connective tissues surrounding the skeletal elements appear to function the same as dermal fibroblasts during regeneration (Gardiner and Bryant, 1989; Muneoka et al., 1986). Given the crucial role of the signaling from nerves and the regeneration epithelium in the induction of dermal fibroblast dedifferentiation and blastema formation, we hypothesize that the same pathways will induce fibroblasts within the center of the limb to dedifferentiate and give rise to multipotential cells that could regenerate an excised skeletal element.

In this paper, we report on a novel model for the regeneration of a critical-size defect (the Excisional Regeneration Model) that can be utilized as an assay to identify signals to induce fibroblast dedifferentiation and skeletal regeneration in salamanders, and potentially in humans. Although this regenerative response is in part mediated by BMP signaling, as is the cases in mammals, a complete regenerative response requires the induction of an undifferentiated, regeneration-competent population of cells. These cells can be generated exogenously by limb amputation leading to blastema cell formation, and can also be generated endogenously in response to signals from a deviated nerve in association with a wound epithelium. The host environment created by excision of the skeletal element is both permissive and instructive for these regeneration-competent cells to replace the missing limb segment.

Materials and methods

Animals and surgical procedures

Experiments were performed on axolotls (*Ambystoma mexicanum*) measuring 8–12 cm from snout to tail tip that were spawned at the University of California, Irvine or the Ambystoma Genetic Stock Center at the University of Kentucky. For all surgeries, we anesthetized animals in a 0.1% solution of MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma), pH 7.0. Animals were kept anesthetized and covered with moist lab tissues for 1 h post-surgery.

To create a mid-diaphyseal radial defect, we made three incisions in the skin overlying the anterior quarter of the lower arm so as to create a skin flap that was still attached to the arm skin on the forth side of the square. We reflected the flap back to expose the underlying soft tissues, and then reflected the muscle fibers to expose the radius. We then used microforceps and iridectomy scissors to dissect the adherent connective tissues, blood vessels and nerves free from the radius and removed a 2-mm segment from the mid-diaphyseal region. We then repositioned the soft tissues and the skin flap, which healed into place without sutures by reepithelialization within 6–8 h (Carlson et al., 1998; Satoh et al., 2008b). To create defects in which a wound epithelium was formed, four incisions were made initially to remove the skin square, and the underlying muscles were removed to expose the radius. After creating the radial defect, we allowed the wound to reepithelialize, which occurs rapidly in these animals, and was complete within 6–8 h (Carlson et al., 1998; Satoh et al., 2008b).

To deviate a nerve to the radial defect, we surgically exposed the pair of nerves that are located between the radius and ulna. We severed these nerves distally and rerouted them to the cavity created by excising the radius. In those experiments in which skin and muscle were removed to induce formation of a wound epithelium, the nerves were positioned such that they eventually were located immediately beneath the wound epithelium after it formed. To test for the requirement for innervation, we denervated limbs by surgically exposing and transecting the third, fourth and fifth brachial nerves distal to the brachial plexus at the scapula level.