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Nerve-dependent and -independent events in blastema formation during *Xenopus* froglet limb regeneration

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Abstract

Blastema formation, the initial stage of epimorphic limb regeneration in amphibians, is an essential process to produce regenerates. In our study on nerve dependency of blastema formation, we used forelimb of *Xenopus laevis* froglets as a system and applied some histological and molecular approaches in order to determine early events during blastema formation. We also investigated the lateral wound healing in comparison to blastema formation in limb regeneration. Our study confirmed at the molecular level that there are nerve-dependent and -independent events during blastema formation after limb amputation, *Tbx5* and *Prx1*, reliable markers of initiation of limb regeneration, that start to be expressed independently of nerve supply, although their expressions cannot be maintained without nerve supply. We also found that cell proliferation activity, cell survival and expression of *Fgf8*, *Fgf10* and *Msx1* in the blastema were affected by denervation, suggesting that these events specific for blastema outgrowth are controlled by the nerve supply. Wound healing, which is thought to be categorized into tissue regeneration, shares some nerve-independent events with epimorphic limb regeneration, although the healing process results in simple restoration of wounded tissue. Overall, our results demonstrate that dedifferentiated blastemal cells formed at the initial phase of limb regeneration must enter the nerve-dependent epimorphic phase for further processes, including blastema outgrowth, and that failure of entry results in a simple redifferentiation as tissue regeneration.

Keywords: Limb regeneration; *Xenopus*; Nerve dependency; Wound healing; Blastema; Tbx5; Prx1; Msx1; Fgf8; Fgf10

Introduction

Blastema formation is an essential process in regeneration of vertebrate appendages, which is a phenomenon that occurs only in amphibians and teleost fishes (reviewed by Brockes and Kumar, 2002; Bryant et al., 2002; Poss et al., 2003; Tanaka, 2003; Tsonis, 2000). In this process, differentiated cells in mature tissues in the amputated organ dedifferentiate into mononuclear blastemal cells that are proliferative and multipotential. The limb blastema in urodele amphibians, which consists of blastemal mesenchymal cells and an overlying wound epidermis (WE; a characteristic skin structure that has no dermal layer), produces all components of a resultant well-patterned regenerate. In contrast to this blastema-mediated regeneration (so-called epimorphic regeneration) of an amputated amphibian limb, differentiated cells in mammals hardly dedifferentiate and/or change their fates under normal conditions, and large-scale removal of limbs of mammals usually results in irregular wound healing with scar formation (Neufeld, 1985, 1989). Limb regeneration in amphibians is one of the most extensively studied phenomena among epimorphic organ regenerations, and each step in the process of limb regeneration, including blastema formation, blastema outgrowth, redifferentiation and repatterning, has been studied mainly using urodele amphibians as materials. Blastema formation, the initial step in limb regeneration, includes some interesting events such as dedifferentiation of mature tissues, promotion of cell proliferation and formation of a WE.

Nerve dependency is a characteristic feature of limb blastema formation. Since discovered by Todd (reviewed by Tsonis, 1996, see also references therein), it has long been known that removal of axons in the limb (denervation) concomitantly with limb amputation inhibits proper blastema formation and outgrowth of the blastema, resulting in the absence of new limb structures. It has been suggested that

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axons secrete neurotrophic factors into the amputation plane in urodele amphibians. In view of results of studies showing that denervation gives rise to an increased rate of apoptosis (Mescher et al., 2000), the neurotrophic factors produced in the neurons are thought to stimulate proliferation and enable blastemal cells to survive (Maden, 1978, 1979; Mescher, 1996; Mescher and Tassava, 1975; Nye et al., 2003). Furthermore, since the expressions of several genes, including *Fgfs* and *Dlx3*, have been reported to be downregulated by denervation (Cannata et al., 2001; Christensen et al., 2001; Mullen et al., 1996), it is thought that neurotrophic factors regulate molecular mechanisms involved in maintenance of blastemal cells and blastema outgrowth. The importance of nerves in regeneration has long been recognized, but little is known about the molecular mechanisms mediating neuronal effects (reviewed by Gardiner et al., 2002).

Studies on nerve deviation that induces blastema-like outgrowth (a bump) to a lateral skin wound of a urodele limb have suggested that neurotrophic factors play an important role in blastema formation itself as well as in its maintenance and outgrowth (Bodemer, 1958, 1959; Endo et al., 2004; Maden and Holder, 1984; Reynolds et al., 1983). Results of those studies also suggest that conservations and differences between limb regeneration and skin regeneration must be important to understand blastema formation and neuronal influences on it. Skin regeneration in amphibians is another example of their regenerative ability. In contrast to imperfect wound healing with scar formation in adult mammals (reviewed by Martin, 1997), amphibians' skin wound healing is perfect. If skin, which includes the epidermis and dermis, is removed, epidermal cells around the wound migrate to cover the exposed mesenchymal tissues and form a WE followed by migration of active dermal fibroblasts under the WE (Endo et al., 2004). Since active dermal fibroblasts express *Hoxd8* and *Hoxd10* (Torok et al., 1998), it is thought that differentiated fibroblasts in mature limb tissues have positional memory as a limb territory where limb regeneration could occur and reactivate the memory in response to injury. However, it is uncertain whether the active fibroblasts during lateral wound healing have the same molecular characteristics as those of dedifferentiated blastemal cells in limb regeneration.

While urodele amphibians can regenerate their appendages perfectly, regeneration ability in anuran amphibians such as *Xenopus laevis* is limited, and a *Xenopus* froglet, a young adult after metamorphosis, merely forms a cartilaginous spike structure on its amputated limb mostly because of deficient ability for pattern formation in the redevelopment phase (Dent, 1962; Endo et al., 2000; Matsuda et al., 2001). However, our previous study (Endo et al., 2000) showed that, in the *Xenopus* froglet blastema, a WE is established, proliferative mesenchymal cells accumulate, and *Msx1*, *Fgf8*, and *Hoxa13*, which are important molecules for limb outgrowth, are expressed. These results suggested that early steps for blastema formation occur normally in *Xenopus* froglets as in urodeles. In addition, there is also nerve dependency of limb blastema formation in *Xenopus* froglets (Endo et al., 2000; Korneluk et al., 1982), while the developing limb bud of early *Xenopus* tadpole can regenerate even if denervated (Filoni and Paglialunga, 1990). It has been reported that effective "uprooting" denervation inhibits spike formation at a high frequency and decreases *Fgf8* expression level in the WE of froglet blastema (Endo et al., 2000). Although *Xenopus* limb regeneration has disadvantages as a model system for epimorphic limb regeneration because of its incomplete ability of repatterning, the initiation of blastema formation in a *Xenopus* limb is thought to be a good model comparable with urodele limb regeneration. Moreover, this organism is advantageous for molecular approaches because of the availability of numerous gene resources, experimental methods at the molecular level and transgenic approaches.

In the present study, we investigated the molecular and cellular aspects of nerve dependency of blastema formation in the *Xenopus* limb. We focused on cellular response (cell proliferation, apoptosis and gene expression) to denervation, with comparison to normal blastema, denervated blastema and lateral wound healing of the *Xenopus* froglet forelimb. First, our observations of cell proliferation and cell death confirmed that characteristics of nerve dependency of *Xenopus* limb blastema formation are comparable to those of urodeles. These observations newly suggest that induction of ectopic apoptosis occurs prior to reduction of proliferative activity in the denervated blastema in *Xenopus*. Moreover, analysis of the expressions of several genes demonstrated that the neurotrophic factors are needed for the maintenance of expression of *Tbx5* and *Prx1* in the early blastema and for the initiation of expression of *Msx1*, *Fgf8* and *Fgf10* in the late blastema. Taken together, the results suggest that the early step for blastema formation consists of some nerve-dependent and -independent events. Generation of blastema cells from differentiated tissues and initiation of an undifferentiated state are likely to be nerve-independent events, and there are some nerve-dependent events, including maintenance of the undifferentiated state and growth/survival of blastemal cells. Interestingly, active fibroblasts in a lateral wound also expressed these genes initially but could not maintain the expressions of these genes at the late stage without denervation, and it therefore seems that the above-described nerve-independent events occurring in an amputated limb also occurred during lateral wound healing in *X. laevis*.

Materials and methods

Animals

X. laevis tadpoles were raised in our laboratory from induced breeding of adult frogs and were allowed to develop until they reached stages 50–56 (Nieuwkoop and Faber, 1956). *X. laevis* froglets (15–20 mm in length from snout to vent) were obtained from local animal suppliers. The animals were kept at 23–24°C in dechlorinated tap water.

Experimental manipulation

Animals were anesthetized in 0.05% ethyl-3-aminobenzoate (Sigma) dissolved in Holtfreter's solution for surgical procedures. For limb amputation, forelimbs of froglets were amputated through the distal zeugopodium,