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Gehan H. Fahmy
University of Minnesota

Raymond E. Sicard
University of Minnesota

ACCELERATION OF AMPHIBIAN FORELIMB REGENERATION BY POLYPEPTIDE GROWTH FACTORS

GEHAN H. FAHMY[1] AND RAYMOND E. SICARD[2]
Regeneration and Organogenesis Section
Center for Wound Healing and Reparative Medicine
University of Minnesota

ABSTRACT

Growth factors are potentially important modulators of epimorphic regeneration. This study examined effects of intraperitoneal administration of selected growth factors on limb regeneration of adult newts, *Notophthalmus viridescens*. These agents stimulated regeneration, producing overlapping but nonidentical effects. Fibroblast growth factor-2 (FGF-2) and insulin-like growth factor I (IGF-I) stimulated bud emergence (8.3 ± 0.6 and 8.3 ± 0.7 days, respectively, vs 11.4 ± 1.1 days for controls). Progression to the cone stage was enhanced by both FGF-2 and transforming growth factor beta 5 (TGF-β_5); 14.6 ± 0.5 and 15.4 ± 0.4 days with FGF-2 and TGF-β_5, respectively, vs 16.5 ± 0.5 days in controls. Insulin accelerated attainment of the palette stage, 17.0 ± 0.7 days vs 19.0 ± 0.4 days for controls. No treatments affected attaining the digital stage; means between 22.4 and 23.4 days. Histological analysis revealed changes consistent with gross observations. In addition, regenerates from newts treated with FGF-2, TGF-β_5, and insulin displayed signs of greater (or earlier) histogenesis than did control animals. These results are consistent with the notion that FGF-2, TGF-β_5, and possibly IGF-I stimulate proliferation of blastema cells and that insulin, FGF-2, and TGF-β_5 promote differentiation and histogenesis during forelimb regeneration. In conclusion, these results demonstrate that several polypeptide growth factors positively affect the progress of forelimb regeneration, that different growth factors influence the same or similar events of epimorphic regeneration, and that diverse growth factors have nonidentical effects on regeneration.

Regeneration of amphibian limbs has long been known to be under neural and endocrine influence.[1] Several investigators have suggested that polypeptide growth factors and hormones mediate these influences. For example, fibroblast growth factors (FGFs) have been shown to stimulate proliferation of blastema cells both in vivo[2] and in vitro.[3,4] More recently, Boilly and associates have demonstrated the presence of FGF-1 in and release by amphibian regeneration blastemas.[5,6] In addition, Poulin and Chiu[7] have documented changes in the distribution of FGF receptors in regenerating newt limbs that suggest a potentially important role for FGFs. A dependence on insulin for progressive regeneration of appendages has also been demonstrated in vitro[8,9] and in vivo.[8,10]

Other growth factors have not yet been implicated as modulators of amphibian limb regeneration. Nevertheless, certain growth factors are likely candidates in view of their roles in skeletal muscle regeneration[11] and wound healing.[12] These factors include the insulin-like growth factors (IGFs) and the transforming growth factor betas (TGF-βs).

Previous studies with FGFs focused on modulating proliferation in bud or cone stage blastemas.[2-4] The observation of widespread and changing distribution of FGF receptors in regenerating limbs[7] suggests that FGFs might be playing a role at other times as well. Furthermore, there is limited or no information pertaining to the role of other polypeptide growth factors on regeneration of amphibian limbs. Accordingly, this study was undertaken to ascertain effects of FGF-2 (basic FGF), IGF-I, insulin, and TFG-β_5 on overall progress of forelimb regeneration in adult *Notophthalmus viridescens* and to suggest events of regeneration that might be modulated or regulated by these factors.

MATERIALS AND METHODS

Adult newts, *Notophthalmus viridescens* (Charles Sullivan, Nashville, TN), were kept in aged deionized water (20 ± 2° C) and fed twice weekly. Regeneration was initiated by bilateral amputations through the distal third of the humerus. Protruding bone was trimmed back to the level of soft tissues. Amputations were performed following anesthetizing in MS-222 (0.1% aqueous methane tricaine sulfonate, pH 7.0; Sigma Chemical Corp., St. Louis, MO).

Experimental treatments consisted of intraperitoneal injections (100 µL) of growth factors every three days from days 6 through 21 postamputation. Treated animals received either recombinant human IGF-I (2000 or 200 ng/injection), bovine brain-derived FGF-2 (340 or 34 ng/injection), recombinant *Xenopus* TFG-β_5 (25 ng/injection) [all from R & D Systems, Minneapolis, MN] or recombinant human insulin (500 ng humulin/injection; Ely Lilly, Indianapolis, IN). Control animals received no treatment.

Gross morphology of regenerating limbs was monitored, with the aid of a dissecting microscope, every other day between days 7 and 25 postamputation. Definition of stages of regeneration was based on descriptions by Iten and Bryant.[13] Representative regenerates from animals in each group were removed for histological study. These tissues were fixed in 10% buffered formalin and decalcified for 7 days in Jenkin's decalcifying mixture. Ten micron paraffin sections were stained with hematoxylin and eosin.

Statistical analysis of data consisted of unpaired t-tests with differences considered significant at $p < 0.05$. Procedures in this study conform to NIH guidelines for animal welfare and were

[1]Present address: Department of Zoology, Faculty of Science, Alexandria University, Moharrem Bey 21511, Alexandria, EGYPT
[2] Address for correspondence: Raymond E. Sicard, Ph.D., Department of Surgery, Box 120 UMHC, University of Minnesota, Minneapolis, MN 55455. Tel: (612) 625-1169; e-mail: Raymond.E.Sicard-1@tc.umn.edu

reviewed by the University Animal Care and Use Committee (University of Minnesota).

Results

The growth factors used positively affected regeneration as expressed by accelerated progression from one stage of regeneration or another (Table 1). While some similarities occurred, effects of the growth factors were not identical. For example, IGF-I and FGF-2 appeared to accelerate bud emergence, causing 78 - 81% and 71 - 88%, respectively, of treated animals to display early buds by 11 days postamputation while only 56% of controls had emerging buds. In addition, 47% of TGF-β_5-treated newts had palette stage regenerates by 17 days postamputation whereas only 22% of controls had reached this stage. Moreover, by 19 days postamputation, 89% of limbs of insulin-treated animals had reached the palette stage (and 28% already had progressed to digital stage) as compared to only 72% of control animals displaying palettes and none with imminent digit formation.

Within 1 - 2 days of initiating treatment, limbs of animals receiving IGF-I or FGF-2 appeared to have enhanced dedifferentiation. This was not seen in limbs of newts receiving TGF-β_5 or insulin. By 15 days postamputation, limbs from animals injected with FGF-2 or TGF-β_5 displayed larger blastemas than controls. More importantly, these limbs seemed to show more differentiation and histogenesis. Limbs from IGF-I- and insulin-treated newts did not display these features. However, limbs from insulin-treated cases displayed similar evidence of histogenesis by 17 days postamputation. In all these latter cases, the degree of redifferentiation and histogenesis exceeded that of control animals. No treatment-related alterations on later histogenesis or patterning were seen during the course of this study.

Discussion

The present study suggests that FGF-2, IGF-I, insulin, and TGF-β_5 can stimulate regeneration of amphibian limbs. However, the influences of these factors on the progress of regeneration were not identical.

FGF-2 and IGF-I seemed to cause blastemas to emerge about two days earlier than controls (Table 1). This appeared to be accompanied by early dedifferentiation. In their study examining the potential of growth factors to substitute for the wound epithelium in sustaining and promoting growth of dedifferentiated cells, Chew and Cameron[14] reported stimulation of mitosis in axolotl limbs implanted with FGF-impregnated beads. Our results are consistent with this effect of FGF. Moreover, our data suggest that IGF might also be able to promote proliferation of dedifferentiated blastema cells. On the other hand, apparent acceleration of blastema emergence also could result from promotion of dedifferentiation itself. However, the design of our study only enables us to raise the question at this time.

FGF-2 and TGF-β_5 appeared to accelerate progression to the cone stage (Table 1), stimulating formation of larger and more developed blastemas than in controls. While it cannot be proved from our data, it is reasonable to suggest that FGF-2 and TGF-β_5 stimulated proliferation to produce these effects. Others have shown that FGFs can stimulate proliferation in blastemas.[2-6] However, our results suggest that TGF-β_5 also might promote proliferation of blastema cells. Further studies are required to confirm this.

Moroever, limbs of newts treated with FGF-2 and TGF-β_5 showed signs of precocious histogenesis. While FGFs are recognized mitogens, they are not generally associated with promoting differentiation. Nevertheless, Chew and Cameron[14] reported precocious chondrogenesis following implantation of FGF-impregnated beads into axolotl limbs. In contrast, TGF-βs have been shown to influence differentiation and histogenesis. For example, TGF-β_1 can promote osteogenesis by mammalian cells in culture; however, it appears to antagonize myogenesis of mammalian myoblasts in vitro.[15] A more detailed understanding of differences between regenerating amphibian limbs and these other models is required in order to reconcile these apparent inconsistencies. While it is premature to suggest specific influences of FGF-2 and TGF-β_5 as direct inducers of skeletogenesis and myogenesis in limb regeneration, such roles would not be uncharacteristic for these factors.

Insulin's influence on regeneration in this study was subtle and late (Table 1). Our results were surprising in light of previous

Table 1 : Effects of growth factors on progressive forelimb regeneration

TREATMENT	DOSE	REGENERATION STAGE (mean days ± 1 sem)			
	(ng/injection)	BUD	CONE	PALETTE	DIGITAL
CONTROL		11.4 ± 1.1	16.5 ± 0.5	19.0 ± 0.4	23.0 ± 0.4
IGF-I	2000	8.3 ± 0.7*	16.1 ± 0.6	18.5 ± 0.6	23.1 ± 0.9
	200	8.7 ± 0.7*	16.6 ± 0.3	19.4 ± 0.5	23.1 ± 1.0
FGF-2	340	9.7 ± 0.7	16.7 ± 0.3	19.2 ± 0.4	22.4 ± 0.4
	34	8.3 ± 0.6*	14.6 ± 0.5*	19.0 ± 0.2	23.4 ± 0.5
TGF-β_5	25	11.8 ± 0.8	15.4 ± 0.4*	18.6 ± 0.7	23.1 ± 0.9
Insulin	500	9.6 ± 0.9	15.0 ± 0.7	17.0 ± 0.7*	22.4 ± 0.8

N = 8 to 10 newts per treatment group (and measurement).
* $p < 0.05$ vs controls.

work[8,9,16] suggesting an important role for insulin in promoting blastemal growth and development. Our data suggest a less important role for insulin in promoting blastemal growth than suggested by these other studies. Differences in the source of insulin used, the dose and method of administration, and other physiological factors could account for this. Additional studies are clearly warranted to reconcile these differences and better determine insulin's role in forelimb regeneration.

In summary, FGF-2, IGF-I, insulin, and TGF-β_5 appeared to promote the progress of forelimb regeneration. Each factor was distinctive in when it seemed to act, alternatively accelerating bud emergence or progression to the cone or palette stages of regeneration. In addition, several events of regeneration (e.g., dedifferentiation, blastemal growth, and histogenesis) appeared to be influenced by one or more of these growth factors. These results underscore that regulation of forelimb regeneration is complex and multifactorial.

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