Nerve-Independence of Limb Regeneration in Larval Xenopus laevis Is Correlated to the Level of fgf-2 mRNA Expression in Limb Tissues

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In both larval and adult urodele amphibians, limb blastema formation requires the presence of an adequate nerve supply. In previous research, we demonstrated that the hindlimb of early Xenopus laevis larvae formed a regeneration blastema even when denervated, while the denervated limb of late larvae did not. We hypothesized that the nerve-independence was due to the autonomous synthesis of a mitogenic neurotrophic-like factor by undifferentiated limb bud cells. In this paper, we demonstrate that fgf-2 mRNA is present in larval limb tissues and that its level is correlated to the extent of mesenchymal cells populating the limb: in early limbs, fgf-2 mRNA is present at high levels all over the limb, while, in late limbs, the fgf-2 expression is low and detectable only in the distal autopodium. After denervation, fgf-2 mRNA synthesis increases in amputated early limbs but not in amputated late limbs. The implantation of anti-FGF-2 beads into amputated early limbs hardly lowers the mitotic activity of blastema cells. However, FGF-2 beads implanted into the blastema of late limbs prevent the denervation-induced inhibition of mitosis and oppose blastema regression. Our data indicate that FGF-2 is a good candidate for the endogenous mitogenic factor responsible for blastema formation and growth in amputated and denervated early limbs. However, in amputated late limbs, the very limited fgf-2 expression is not sufficient to promote blastema formation in the absence of nerves. © 2001 Academic Press

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INTRODUCTION

Many species of urodele amphibians are able to regenerate their limbs not only at larval stages but also as adults. In both the larva and the adult, urodele limb regeneration requires the presence of an adequate nerve supply. When the number of nervous fibers present in the limb stump is below a threshold level, a regeneration blastema does not take place (see Wallace, 1981). Several experimental evidences support Singer’s hypothesis (Singer, 1965) that the nerve fibers release a trophic substance (neurotrophic factor), which is necessary for the proliferation of blastemal cells (see Wallace, 1981; Tassava and Olsen, 1985). Blas-
stimulate the limb cells to begin synthesizing the non-
nervonal trophic factor again or would lower the threshold
level of innervation required by the limb stump tissues for
blastema formation.

Anuran amphibians can completely regenerate ampu-
tated limbs only during early larval stages. This capacity
decreases proximo-distally as the larva develops, and gen-
gernally disappears at metamorphosis. Limb structures of
Xenopus laevis do not regenerate after metamorphosis, and a
hypomorphic outgrowth occurs following amputation
(Schött and Harland, 1943; Dent, 1962). It was recently
observed that, contrary to urodeles, the hindlimbs of early
X. laevis larvae regenerate even when denervated and that
nerve-dependence for blastema formation is acquired dur-
ing larval stages, in a proximal to distal direction, as limb
development and differentiation proceed (Filoni and Pagli-

alunga, 1990). In fact, denervated limbs of stage 51 larvae
(according to Nieuwkoop and Faber, 1956) form regenera-
tion blastemas after distal and proximal amputation, while
those of stage 55 form regeneration blastemas only after
distal amputation; denervated limbs of stage 57 larvae never
form regeneration blastemas. Further data obtained both
after transplantation of early and late limbs onto denervated
limbs and after in vitro culture of early and late amputated
limbs have shown that nerve-independence of early limbs
and nerve-dependence of late limbs are due to intrinsic
factors of the limb tissues (Filoni et al., 1991b; Cannata
et al., 1992). It could be supposed, in accordance with Singer's
hypothesis, that the nerve-independence of hindlimbs of X.
laevis larvae could be due to the autonomous synthesis of
neurotrophic-like factors by the limb bud cells, the nerve-
dependence being acquired as the cells gradually stop pro-
ducing such factors following an inhibitory effect of factors
released by the nerves. Further experiments indicate that,
contrary to Singer's hypothesis for urodeles, in larval X.
laevis the nerve-dependence for blastema formation is not
imposed by factors released by the nerve fibres, but it is
related to the differentiation of limb tissues. In fact, the
capacity to form a nerve-independent blastema is main-
tained in early limbs that have been richly innervated, but
is lost in late limbs differentiated in the absence of nerves
(Filoni et al., 1995). However, according to Singer's hypoth-
thesis, several data indicate that promoting factors of non-
nervous origin, but similar in action to those of nervous
tissue, are indeed present in early limbs and absent in late
limbs (Filoni et al., 1991b; Filoni et al., 1999). In particular,
we observed that the environment of denervated early
limbs induced the transdifferentiation of corneal fragments
into lens fibers, as did the neural retina during in situ lens
regeneration, but the environment of denervated late limbs
did not (Filoni et al., 1991a). Moreover, hindlimb segments
of early limbs, grafted into denervated nerve-dependent
blastemas of late larval limbs or of postmetamorphic limbs,
removed the denervation-derived inhibition of DNA syn-
thesis and mitosis of blastemal cells and prevented the
reabsorption of blastemas of postmetamorphic limbs. These
effects were comparable to those exerted by the grafting of
spinal cord segments. On the contrary, implants of late
hindlimb segments were completely ineffective (Filoni
et al., 1999). These data suggest that the undifferentiated cells
of early limb tissues produce and release a diffusible factor
that imitates the one released by nerve tissues. As the larva
develops, the progressive decrease in undifferentiated cell
population in limb tissues would cause a decrease in the
level of tissue mitogenic factor and would establish depend-
ance on the factor released by the nerves.

Research conducted on the urodele limb regeneration
indicates that the neural influence involves general nutritive
factors and more specific factors. Good candidates are
transferrin (Tf), the glial growth factor (GGF), and the acidic
and basic fibroblast growth factors (FGF-1 and FGF-2, re-
spectively) since they were shown to be mitogenic for
blastema cells (Mescher and Loh, 1981; Brockes and Kint-
ner, 1986; Albert et al., 1987; Albert and Boilly, 1988;
Boilly et al., 1991; Zenjari et al., 1996; Mescher et al., 1997).
By implanting slow-release resin micropersules imbibed with
FGF-2 into nerve-dependent regeneration blastemas of axo-
lotl limbs, Mullen et al. (1996) showed that FGF-2 can
re-establish the expression of several genes, which had been
inactivated after denervation, and thus allowing denervated
limbs to regenerate. Since FGF-2 was the first substance
identified to permit the regeneration of a denervated
urodele limb, the authors suggested that FGF-2 was, in fact,
the neurotrophic factor. However, Wang et al. (2000) ob-
served a regenerative response after injections of recombi-
nant human GGF2 into denervated, nerve-dependent regen-
eration blastemas of axolotl limbs.

In this paper, we tested our previous hypothesis on
nerve-independent limb blastema formation in larval X.
laevis by investigating the presence, the cellular source, and
function of FGF-2 in X. laevis limb tissues during larval
development. We analyzed, by using RT-PCR and whole-
mount in situ hybridization analysis, the level and the
expression pattern of fgg-2 mRNA in normal and amputated
limbs, both innervated and denervated, of larvae at various
stages of development. Moreover, to investigate whether
the fgg-2 mRNA produced by the limb tissues was trans-
lated into protein, we analyzed its association with poly-
somes. Finally, by implantation of FGF-2- or anti-FGF-2-
socketed beads into amputated limbs, we tested the function
of FGF-2 in limb blastema formation and growth.

MATERIALS AND METHODS

Animal Procedures

The X. laevis larvae used in this study were spawned in our
laboratory according to the method of Nieuwkoop and Faber (1956).
Tadpoles were reared to the appropriate stages in dechlorinated tap
water at 23 ± 1°C, maintained on 12/12 h photocycle, and fed on
nettle powder. After metamorphosis, froglets were fed on chopped
beef heart. The experiments were performed on larvae at stages 52,
55, and 57 (according to Nieuwkoop and Faber, 1956). Specimens
were anaesthetized in MS 222 (tricaine metasulphonate, Sigma) at
concentrations of 1:3000 and operated under a binocular stereo-
Whole-Mount in Situ Hybridization

Whole-mount in situ hybridization was performed as in Harland (1991) with modification as in Pownall et al. (1996). Larvae at stages 52, 55, and 57 (45 for each stage) were anaesthetized in MS 222 and fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) for 1 h at room temperature and stored in 100% ethanol at −20°C until further processing. Larvae were rehydrated through a graded series of ethanol/PBS and then rinsed in PBS with 0.1% Tween 20 (Sigma). Proteinase K treatment was performed for 20 min at room temperature with 10 μg/ml of Proteinase K. Hybridization was carried out overnight at 60°C in 50% formamide, 5× SSC, 1 mg/ml total RNA from yeast, 100 μg/ml heparin, 1× Denhart’s, 0.1% Tween, 0.1% CHAPS, 10 mM EDTA. Extensive washes in 2× SSC, and 0.2× SSC at 60°C were followed by washes at room temperature with maleic acid buffer, 50 mM NaCl, 50 mM NaAc, 0.1% Tween, pH 7.8) and blocking in 2% Boehringer Mannheim Blocking Reagent and 20% heat-treated lamb serum for 2 h at room temperature. Larvae were then incubated with anti-DIG AP-conjugated antibody at dilution of 1/2000 in blocking solution at 4°C overnight. The antibody was detected after extensive washes at room temperature in MAB and two washes in alkaline phosphatase buffer (100 mM Trizma, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween, 5 mM Levamisole) by a color reaction using BM purple (plus 5 μM Levamisole) precipitating alkaline phosphatase detection system (Boehringer Mannheim). Probes for in situ hybridization were transcribed using DIG RNA labeling mix (Boehringer Mannheim), according to the manufacturer, from linearized plasmids (for fgf-8, Xfgfb8s, linearized with XbaI and transcribed with T3 RNA polymerase for antisense; for fgf-2, XF62, linearized with EcoRI and transcribed with SP6 RNA polymerase for antisense; for Pax6, XPax6-FLAG G5S2+, linearized with EcoRI and transcribed with T3 RNA polymerase for antisense). To maximize the efficiency of probes, they were hydrolyzed to approximately 300 bases using 80 mM NaHCO3, and 120 mM Na2CO3 (Wilkinson and Green, 1990). Digoxigenin-labeled antisense riboprobes for fgf-8 (that labels specifically developing limbs; Christen and Slack, 1997) and for Pax-6 (absent in developing limbs; Hirsch and Harris, 1997), were used as controls of whole-mount in situ hybridization procedures. Moreover, some embryos at stage 35 fixed and processed for whole-mount in situ hybridization as aforesaid described were used as positive control for fgf-2 mRNA probe expression.

RT-PCR Analysis

To detect the low level of fgf-2 mRNA expression in limbs of early and late larvae, we performed a radioactive RT-PCR analysis. The RT-PCR analysis was carried out by utilizing developing normal limbs and regenerating limbs, either innervated or denervated dissected from stage 52 and 57 larvae at the base of the thigh or the middle-shank, respectively. For each RT-PCR analysis, 10 limbs from each batch were homogenized in SDS 1% and Proteinase K 1 mg/ml for RNA extraction. After 30 min at 37°C, the limb tissues were further homogenized and total RNA extracted with phenol/chloroform and ethanol precipitated in presence of 10 μg of glycogen.

RNA samples extracted from limb tissues were DNAase-treated, and each sample was reverse-transcribed by the random hexanucleotides technique using 100 U of M-MLV RTase (GibcoBRL) according to the manufacturer. An aliquot of RT reaction was PCR-amplified in a final volume of 50 μl, by using 20 pmol of each primer, 200 μM of dATP, dCTP, and dTTP, 10 μM of dCTP, 0.2 μCi of [α-32P]dCTP (Amersham, 3000 Ci/mM) and 0.5 U of Taq DNA Polymerase (Pharmacia). As quantitative amplification was required, the amount of template and the number of amplification cycles were optimized for each PCR to avoid conditions of saturation. We used the house-keeping gene ornithine decarboxylase (ODC) as internal standard because this gene is uniformly expressed during development (Bassez et al., 1990). For ODC mRNA amplification, ODC(5′) (5′ CACATGTCAGCCAGTTC 3′) and ODC(3′) (5′ GCCATACATTGATCTG 3′) were used as primers giving an amplification fragment of 381 bp.

For fgf-2 mRNA amplification, we used FGFI(1′) (5′ CGCGGAATTCGTCATTCTTGGC 3′) and FGFI(4′) (5′ CGGAAGCCTGTACTCCCT 3′) as primers; the product was a fragment of 320 bp.

The cycling reactions were performed with a DNA Thermal Cycler (PCR) and Hybrid. The first cycle was at 94°C for 3 min, followed by 27 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, for ODC mRNA amplification and 30 cycles for fgf-2 mRNA amplification. One-fifth of the PCR was analyzed on 5% acrylamide gel, transferred on 3MM paper and exposed to X-Ray film and quantitatively analyzed by a PhosphorImager (Molecular Dynamics).

The amplified fgf-2 product was cloned and sequenced to confirm the correspondence to the fgf-2 sequence.

The fgf-2 mRNA values were expressed as % of ODC mRNA (considered as internal control). In each experiment, all data were expressed as the mean ± standard deviation (SD) of three different pools made up of 10 limbs each, and differences in mean between stages 52 and 57 limbs were analyzed for significance by Student’s t-test.

Polysomes/RNPs Distribution of fgf-2 mRNA

This assay was performed by sucrose gradient fractionation of a cytoplasmic extract followed by radioactive RT-PCR.

Extract preparation, sucrose gradient sedimentation of polysomes, and analysis of the polysomes-RNPs distribution of mRNAs were carried out according to Bagni et al. (2000). Briefly, amputated and sham denervated limbs or amputated and denervated limbs from stage 52 larvae sacrificed 3 days after operations were homogenized, and lysis were carried out in lysis buffer (10 mM NaCl, 10 mM MgCl2, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% Sodium deoxycholate, 1 mM Dithiothreitol, 30 U/ml RNase Inhibitor (Promega) and 100 μg/ml cycloheximide). After 5 min of incubation on ice, the lysates were centrifuged for 5 min at 12,000g at 4°C. The supernatants were frozen in liquid nitrogen and stored at −70°C or immediately sedimented in a 5-70% (w/v) sucrose gradient by centrifugation for 135 min at 37,000g in a Beckman SW41 rotor. Each gradient was collected in nine fractions while monitoring the absorbance at 254 nm. RNA extracted from gradient fractions was analyzed by RT-PCR as previously described. To minimize experimental errors due to variations in the RT and PCR, an equal amount of a synthetic RNA was added to each sample and used as an amplification control. This RNA was
obtained by in vitro transcription (Ambion) of the Xenopus ribosomal protein L27A sequence (formerly L22, accession number X64207), and detected with specific oligonucleotides.

**Implantation of Acrylic Beads**

Heparin acrylic beads (Sigma), of 200–250 μm in diameter, were washed in PBS and incubated, for 12 h at 4°C, in a rabbit polyclonal neutralizing antibody against bovine FGF-2 (Sigma, product No. F 3393), or in 0.2 μg/μl bovine FGF-2 (Sigma, product No. F 3133). Beads incubated in rabbit IgG or in bovine albumin (BSA) were used as control. The beads were washed in PBS just prior to implantation.

**Experiment I: Bead Implantation in Stage 52 Regenerating Limbs**

The right hindlimbs of 15 larvae at stage 52 were amputated through the tarsalia. Ten of them were also denervated, while the other five were sham-denervated. After 1 day, an anti-FGF-2 bead was inserted beneath the wound epithelium of five denervated limbs, while a bead soaked in rabbit IgG was implanted into the other five denervated limbs and in the five sham-denervated limbs. Four days after bead implantation (5 days after amputation), the larvae were sacrificed and fixed in Bouin’s liquid.

**Experiment II: Bead Implantation in Stage 57 Regenerating Limbs**

The right hindlimbs of 25 larvae at stage 57 were amputated through the tarsalia. Six days after amputation and upon examination with a dissection microscope, 18 limbs presenting a mound blastema were selected. Twelve of these limbs were denervated, while the remaining six were sham-denervated. Immediately after these operations, three to five FGF-2-soaked beads were inserted into the regeneration blastema of six denervated limbs, while BSA-soaked beads were inserted in the blastema of the other six denervated limbs and in the blastema of the six sham-denervated limbs. Four days after bead implantation (10 days after amputation), the larvae were sacrificed and fixed in Bouin’s liquid.

**RESULTS**

**Whole-Mount in Situ Hybridization (Fig. 1)**

**Analysis of fgf-2 expression in developing limbs.** In normal stage 52 larvae, the hindlimb is still largely undifferentiated. In the skeleton, only the femur is condensing, while the other parts of the limb are indicated as separate mesenchymal condensations. Local condensations of mesenchymal cells indicate muscle formation in the thigh and leg. At this stage, a high level of fgf-2 mRNA expression is detected all over the hindlimb mesenchyme, especially in the intermediate and distal parts of the limb (Fig. 1A). At stage 55, the femur, tibia, and fibula are well chondrified and perichondrial ossification begins in the middle region of the femur; in the autopodium, tarsalia and metatarsalia are condensing, but the phalanxes are still procartilaginous or mesenchymatous. Muscle cells are beginning to differentiate. Mesenchymal cells, however, are still abundant. At this stage, fgf-2 expression appears to be graded proximodistally, with the highest levels in the autopodium mesenchyme (Fig. 1B).

At stage 57, perichondrial ossification is observed in the femur, tibia, and fibula and in the proximal elements of autopodium; in the distal autopodium, most of the phalanxes are cartilaginous and undifferentiated mesenchymal cells are still fairly abundant. Muscles are well-formed and functional. At this stage, a low level of fgf-2 expression is still detectable in the distal autopodium (in the mesenchyme bordering the phalanxes), but it is not observed in the zeugopodium and stylopodium (Fig. 1C).

**Analysis of fgf-2 expression in amputated and sham-denervated limbs.** After amputation at stages 52 and 55, a mound blastema covered by a thickened wound epithelium was evident 3 and 4 days after operation, respectively. At both stages, the regeneration blastema grew rapidly and became cone-shaped 5 and 6 days after operation. In the limb of stage 52 and 55 larvae, sacrificed 3 and 5 days after operation, an intense expression of fgf-2 mRNA was observed both in the stump and in the regeneration blastema (data not shown).

After amputation at stage 57, a mound blastema formed 6 days after operation and attained the early cone stage between days 7 and 9. In the limb of stage 57 larvae, a high level of fgf-2 expression was detected only in the most distal part of the stump, during the wound healing and early dedifferentiation stages (3 days after operation) (Fig. 1G). In the following days, a sharp decrease in the level of fgf-2 expression was observed, and 9 days after operation, no fgf-2 expression was detectable in the stump and in the growing blastema (Fig. 1H).

**Analysis of fgf-2 expression in amputated and denervated limbs.** At stages 52 and 55, a regeneration blastema formed. The days taken to attain the mound blastema and the conic blastema stages were superimposable on those for the amputated and sham-denervated limbs. Three and five
Whole-mount in situ hybridization with fgf-2 mRNA (A–I) and fgf-8 mRNA antisense probes (L, M). (A–C) Normal hindlimbs from larvae at stages 52, 55, and 57, respectively. (A and B) At stage 52 and 55, fgf-2 mRNA expression is detectable in whole the limb, but a distal to proximal gradient of the fgf-2 expression pattern is visible. (C) At stage 57, a low level of fgf-2 mRNA is observed only at level of the distal autopodium (toes). (D–F) Regenerating hindlimbs amputated through the tarsalia and denervated, stage 52. (D and E) Three and five days after operation, respectively. A high level of fgf-2 mRNA expression is observed both in the stump and in the regeneration blastema. Red lines indicate the approximate amputation level. (F) Longitudinal section of the same blastema in E. fgf-2 mRNA is confined to mesenchymal cells in the stump and in the regeneration blastema (arrowheads). In the procartilaginous skeleton of the stump (arrow), fgf-2 mRNA is undetectable. The line marks the approximate boundary between the stump and regeneration blastema. (G–I) Hindlimbs amputated through the tarsalia, stage 57. (G) Three days after amputation and sham-denervation. fgf-2 mRNA expression is evident only in the most distal part of the stump in close proximity to the amputation surface. (H) Nine days after amputation and sham-denervation. fgf-2 mRNA is undetectable both in the stump and in the blastema (arrow). (I) Nine days after amputation and denervation. No blastema is present and fgf-2 mRNA is undetectable in the stumped limb. (L and M) fgf-8 expression in hindlimbs of stage 52 larvae. In the normal limb (L), fgf-8 is expressed in the distal epidermis. In the regenerating limb (M), fixed 4 days after amputation, fgf-8 is expressed in the apical epithelial cap covering the regeneration blastema. rb, regeneration blastema; st, stump. Scale bar, 500 μm.
days after operation, a high level of fgf-2 expression was observed both in the stump and in the blastema (Fig. 1D and 1E), as observed in amputated and sham-denervated limbs. The fgf-2 expression was localized in the mesenchymal cells present in undifferentiated regions of the stump and in the regeneration blastema (Fig. 1F).

At stage 57, no regeneration blastema formed. However, in accordance with our previous observations (Filoni and Paglialunga, 1990), the early phases of the regenerative process, consisting of the formation of wound epithelium and a little accumulation of dedifferentiated mesenchymal cells beneath it, took place in a similar way to the controls. Between days 6 and 10, the mesenchymal cells were replaced by a thick fibrous connective tissue pad. During the wound healing and early dedifferentiation stages (3 days after operation), a high level of fgf-2 expression was observed only in the most distal part of the stump, as in sham-denervated limbs (not shown). In the following days, a sharp decrease in the level of fgf-2 expression was observed, and, 9 days after operation, no fgf-2 expression was detectable in the stumped limbs (Fig. 1I).

In control embryos at stage 35, the expression pattern of fgf-2 transcript was superimposable to that previously described by Song and Slack (1994), i.e., in the brain, eyes, ears, and branchial arch mesenchyme and, at a lower level, along the myotomes (not shown).

Controls of the whole-mount hybridization procedure carried out by using fgf-8 and Pax6 probes showed that the fgf-8 hybridization pattern corresponded to that previously described by Christen and Slack (1997), i.e., fgf-8 was expressed in the distal epidermis of normal and regenerating early limbs (Fig. 1L and 1M). However, Pax6, which is specifically expressed in the embryo CNS (Hirsch and Harris 1997), was not detectable in the limb tissues.

RT-PCR Analysis (Fig. 2)

Levels of fgf-2 mRNA in developing limbs. In limbs dissected from stage 52 larvae at the base of the thigh, the RT-PCR analysis showed that the fgf-2 mRNA level was significantly higher (P < 0.05) than the level observed in limbs dissected from stage 57 X. laevis larvae sacrificed 3 days after operation. After amputation and denervation, fgf-2 mRNA level increases in early limbs but not in late limbs. Each column represents the mean value ± SD obtained from RT-PCRs performed on three different pools of ten limbs.

Levels of fgf-2 mRNA in amputated and sham-denervated limbs. Three days after amputation, the level of fgf-2 mRNA underwent a significant increase (P < 0.05) in early limbs but not in late limbs.

Levels of fgf-2 mRNA in amputated and denervated limbs. Three days after amputation and denervation of early limbs, the fgf-2 level was threefold higher when compared with the level observed in amputated and sham-denervated early limbs of Experiment I (P < 0.001). However, in amputated late limbs, no significant difference in fgf-2 level of denervated and sham-denervated limbs was observed.

Polysomes/RNPs Distribution of fgf-2 mRNA (Fig. 3)

The results obtained from cytoplasmic extracts of amputated limbs from stage 52 larvae showed that fgf-2 mRNA produced by cells of both simply amputated limbs and amputated and denervated limbs was actively translated.

Implantation of Acrylic Beads

Experiment I: Bead implantation in stage 52 regenerating limbs. The MI of denervated blastemas implanted with anti-FGF2-soaked beads was significantly lower than
the MI of both denervated and sham-denervated blastemas implanted with IgG soaked beads (P < 0.001) (Fig. 4).

Experiment II: Bead implantation in stage 57 regenerating limbs. The MI of denervated blastemas implanted with FGF-2-soaked beads was significantly higher than the MI of denervated blastemas implanted with BSA-soaked beads (P < 0.001) and superimposable to the MI of sham-denervated blastemas implanted with BSA-soaked beads (Fig. 4). Moreover, while the denervated blastemas implanted with BSA-soaked beads were regressing, those implanted with FGF-2-soaked beads were still well developed (Fig. 5).

DISCUSSION

This research sheds light on a developmental phenomenon evidenced for the first time by some of us (Filoni and Paglialunga, 1990) showing that, contrary to the general rule according to which amphibian limb regeneration depends on nerves, the hindlimb of X. laevis larvae can regenerate in the absence of nerves becoming nerve-dependent only at the end of the larval life. Present results support our previous hypothesis that the nerve-independence for blastema formation and growth is due to the synthesis of a mitogenic neurotrophic-like factor by undifferentiated cells of early limb tissues and that the onset of the nerve-dependence is related to the gradual loss of the capacity to produce this factor as the limb tissues differentiate.

fgf-2 mRNA Is Detectable in the Mesenchyme of Developing Limbs

The results of PCR and in situ hybridization of normal limbs show that fgf-2 mRNA is present in developing limbs of larval X. laevis and that its level and expression pattern are correlated to the extent and localization of undifferentiated mesenchymal cell population present in the limb.
tissues. In early limbs (stage 52), where mesenchymal cells are quite abundant also in the proximal part of the limb, fgf-2 mRNA is present at high level and distributed all over the limb, although a more intense expression is observed in the distal part. In stage 55 limbs, where mesenchymal cells are still abundant in the distal part, decreasing gradually in proximal direction, the fgf-2 expression pattern shows a more evident distal to proximal gradient. Finally, at stage 57, where differentiation is underway also in the autopodium, the fgf-2 expression level is low and fgf-2 mRNA is detectable only in the distal autopodium. A great number of evidences in chick and mouse embryos have shown that FGFs are signaling molecules that are important in growth control and patterning of the vertebrate limb development. Signals including FGF-2 and FGF-10 from the mesenchyme (Savage and Fallon, 1995; Ohuchi et al., 1997; Xu et al., 1998) and FGF-8 from the prospective limb bud ectoderm (Mahmood et al., 1995; Vogel et al., 1996) serve as limb bud initiators and promote either directly or indirectly the formation of a functional apical ectodermal ridge (AER), the permissive epidermis located at the tip of the limb bud ectoderm. Once the limb bud is formed, FGF-2, FGF-4, and FGF-8 from the prospective limb bud ectoderm (Savage and Fallon, 1995; Cox et al., 1993; Cohn et al., 1994; Xu et al., 1994; Heikinheimo et al., 1994; Martin, 1998). In fact, in FGF-10-deficient mice, the AER does not form (Sekine et al., 1995; Vogel et al., 1996) serve as limb bud initiators and promote either directly or indirectly the formation of a functional apical ectodermal ridge (AER), the permissive epidermis located at the tip of the limb bud ectoderm. Once the limb bud is formed, FGF-2, FGF-4, and FGF-8 are expressed in the AER and sustain the outgrowth and patterning of the limb mesenchyme of developing chick and mouse embryos (Niswander and Martin, 1992; Savage et al., 1993; Heikinheimo et al., 1994; Martin, 1998). In fact, in FGF-10-deficient mice, the AER does not form (Sekine et al., 1999) and exogenous FGF-2, FGF-4, and FGF-8 can substitute for the AER and induce formation of ectopic limb buds in the flank of chick embryos (Fallon et al., 1994; Niswander et al., 1993; Vogel et al., 1996; Cohn et al., 1995). In larval Xenopus, a prominent expression of fgf-8 has been observed at the distal tip of the outgrowing limb bud along the dorsal–ventral boundary (Christen and Slack, 1997). Although in the Xenopus limb bud there is no morphological AER, this band of expression suggests that the Xenopus limb bud contains a cryptic region specialized to secrete fgf-8 and fulfill the same function of AER in the amniote limb bud (Christen and Slack, 1997). The FGF-2 produced in the mesenchyme of larval Xenopus limb buds could stimulate limb growth by inducing and/or promoting a functional AER secreting FGF-8. An investigation is currently underway in our laboratory with the aim of analyzing the relationship between fgf-2 produced in the mesenchyme and the expression of fgf-8 in the distal epidermis of Xenopus limbs.

After Denervation, fgf-2 mRNA Synthesis Increases in Amputated Early Limbs but Not in Amputated Late Limbs

Among members of FGF family, FGF-1, FGF-2, and FGF-8 have been detected in nerves and/or in regenerating limbs. FGF-1 is present in nerves (Elde et al., 1991), in the apical epithelial cap (AEC), the permissive epidermis that promotes blastema growth and pattern formation (Stocum and Dearlove, 1972), and in the blastema mesenchyme of regenerating newt limbs (Boily et al., 1991). Mullen et al. (1996) detected the presence of FGF-2 in the AEC and in nerves of regenerating axolotl limbs and found that exogenous FGF-2 could restore the expression of Dlx-3 (a homologue of Drosophila Dll required for leg development; Cohen et al., 1989), and FGF-2 synthesis in the AEC of denervated blastemas. Moreover, re-expression of Xfgf-8 in the AEC has been observed in regenerating hindlimbs of larval X. laevis (Christen and Slack, 1997). The current hypothesis is that FGF-1 and/or FGF-2 released from damaged and regenerating nerves induce the formation of the permissive epidermis that in turn produces several form of FGF promoting growth in the limb mesenchyme and pattern formation (Poulin et al., 1993; Mullen et al., 1996). Recently, Endo et al. (2000) observed a correlation between the presence of a connective tissue pad in denervated, not regenerating, limbs of X. laevis froglets, and a minimal fgf-8 expression suggesting that the dermal layer may disturb the epidermal–mesenchymal interaction, resulting in the failure of fgf-8 expression. Moreover, Yokoyama et al. (2000) showed that, in larval X. laevis, fgf-10 expression in the limb mesenchyme corresponded to the regenerative capacity and that fgf-10 and fgf-8 were synergistically expressed in regenerating blastemas.

The results from PCR, in situ hybridization, and analysis of fgf-2 mRNA associated with polysomes show that, in regenerating limbs of early larvae, both innervated and denervated, the fgf-2 mRNA is present in the mesenchyme of the stump and in the regeneration blastema and is translated into protein. This indicates that, in these limbs, there is a FGF-2 of local origin. Since, after denervation, the level of fgf-2 mRNA and its translation in amputated early limbs increases significantly, it is likely that the synthesis of FGF-2 in the limb mesenchyme is under the control of FGF-2 released from nerves. Due to upregulation of fgf-2 expression in response to denervation, sufficient FGF-2 would be produced by the mesenchymal cells to promote blastemal cell proliferation and to allow a nerve-independent regeneration. In amputated limbs of late larvae, however, fgf-2 expression is detectable only in the proximity of the amputation surface, during the wound healing and early dedifferentiation stages. This spatially and temporally localized fgf-2 expression persists both in normal and denervated late limbs only a few days. Considering that FGFs are also involved in angiogenesis and tissue repair (Greenhalg et al., 1990; Basilico and Moscatelli, 1992) and that denervated late limbs did not form a regenerating blastema, our results indicate that the fgf-2 expression at the level of the amputation surface of late limbs is related to wound healing and is not sufficient to promote blastema formation in the absence of FGF-2 released from the nerves.
Antibodies to FGF-2 Inhibit Blastema Cell Proliferation in Denervated Early Limbs, while Exogenous FGF-2 Rescues Blastema Cell Proliferation and Opposes Blastema Regression in Denervated Late Limbs

The results obtained in Experiments I and II show that anti-FGF2-containing beads implanted beneath the wound epithelium of amputated early limbs hardly lower the mitotic activity of blastema cells; however, FGF2-containing beads implanted into denervated blastemas of late limbs prevent the denervation-induced inhibition of mitosis and blastema regression. Interestingly, these effects were very similar to those exerted by the grafting of spinal cord segments into denervated blastemas of late limbs (Filoni et al., 1999).

On the whole, data obtained in the present research show that there is a complete correlation between the ability of larval X. laevis limbs to form a regeneration blastema in the absence of nerves and the FGF-2 synthesis in the limb mesenchyme; moreover, FGF-2 has a critical role for blastema proliferation and maintenance. Thus, present data indicate that FGF-2 is a good candidate for the endogenous mitogenic factor responsible for nerve-independent blastema formation and growth in early limbs of larval X. laevis, although the possibility that other growth factors may have a similar function is not excluded.

The FGF-2 produced in the limb mesenchyme could act as an autocrine factor stimulating blastemal cell proliferation and as a paracrine factor promoting in the permissive epithelium. It would appear, then, that the wound epithelium. On the whole, data obtained in the present research show that there is a complete correlation between the ability of larval X. laevis limbs to form a regeneration blastema in the absence of nerves and the FGF-2 synthesis in the limb mesenchyme; moreover, FGF-2 has a critical role for blastema proliferation and maintenance. Thus, present data indicate that FGF-2 is a good candidate for the endogenous mitogenic factor responsible for nerve-independent blastema formation and growth in early limbs of larval X. laevis, although the possibility that other growth factors may have a similar function is not excluded.

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