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Original Article

Experimental evidence for FGF-1 control of blastema cell proliferation during limb regeneration of the Amphibian *Pleurodeles waltl*

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ABSTRACT During regeneration, blastema cell proliferation depends on several different factors which are, as yet, not fully understood. Previous studies showing the presence of FGF-1 and FGF receptors in the limb blastema make FGF-1 a potentially important molecule for limb regeneration but they do not demonstrate that this factor is active during the process. In the present study, we have first of all confirmed the presence of FGF-1 in limb blastemas of the amphibian *Pleurodeles waltl* using immunochemistry. Second, we provide evidence *in vivo* that FGF-1 controls blastema cell proliferation by using different reagents which interfere with FGF activity. Sulfated polysaccharides which bind FGFs, such as heparin, ι-carrageenan and pentosan polysulfate, are able to decrease both ^{3}H-thymidine incorporation and the mitotic index in regeneration blastemas. In addition, suramin which inhibits the binding of growth factors to their receptors, induces the same effect. The presence of receptors in blastema cells is also demonstrated by using the FGF-saporin complex which is known to bind to FGF receptors and to kill cells bearing these receptors. This complex decreases the mitotic index in mesenchyme, while saporin alone did not influence cell proliferation. Finally, results obtained using a neutralizing monoclonal antibody against FGF-1 which was able to specifically reduce blastema cell proliferation, suggests that FGF-1 plays an important function in limb regeneration.

KEY WORDS: *limb regeneration, FGF, sulfated polysaccharides, suramin, cell proliferation, Amphibians*

Introduction

Some amphibians (Urodeles and larvae of Anurans) have the ability to regenerate their limbs after amputation (Wallace, 1981; Chernoff and Stocum, 1995; Tsonis, 1996 for review). The process of limb regeneration resembles limb development at least at the morphological level (Faber, 1971). After wound healing, a blastema arises at the surface of the limb stump; the blastema is composed of an epidermal cap, which covers a mass of dedifferentiated mesenchymal cells (i.e. blastema cells) that proliferate very actively. After this phase of strong cell proliferation, the blastema differentiates and restores the original limb pattern.

It is known that limb regeneration, as well as limb development, depends on proliferation of mesenchymal cells. During regeneration, blastema cell proliferation depends on different factors, some of them originating both from the severed peripheral nerves and from the epidermal cap (Boilly *et al.*, 1990). Nevertheless, the nature of factors which trigger blastema cell proliferation remain unknown. Several mitogens, assumed to be produced by nerves, such as substance P (Globus and Vethamany-Globus, 1985; Smith *et al.*, 1995), transferrin (Mescher and Munaim, 1984) and fibroblast growth factor (FGF) (Gospodarowicz and Mescher, 1980), were considered to act during limb regeneration. FGF received particular attention especially at a time when this growth factor was first purified from nerve tissues (Gospodarowicz, 1975). Currently the family of FGFs consists of 9 structurally-related polypeptides: the first two represent the well-known prototypes, FGF-1 (acidic FGF) and FGF-2 (basic FGF) (review in Fernig and Gallagher, 1994). They act through high-affinity tyrosine kinase receptors and low affinity binding sites; the latter are heparan sulfate proteoglycans (HSPG) present both on the cell surface and in the extracellular matrix (ECM). HSPG plays an important role in FGF function. They are known to bind FGF on their heparan sulfate moieties and to present it to FGF receptors, to protect FGF-1 from denaturation and also to modulate the effect of FGF by a competition process.

Abbreviations used in this paper: ECM, extracellular matrix; FGF, fibroblast growth factor; HSPG, heparan sulfate proteoglycan.

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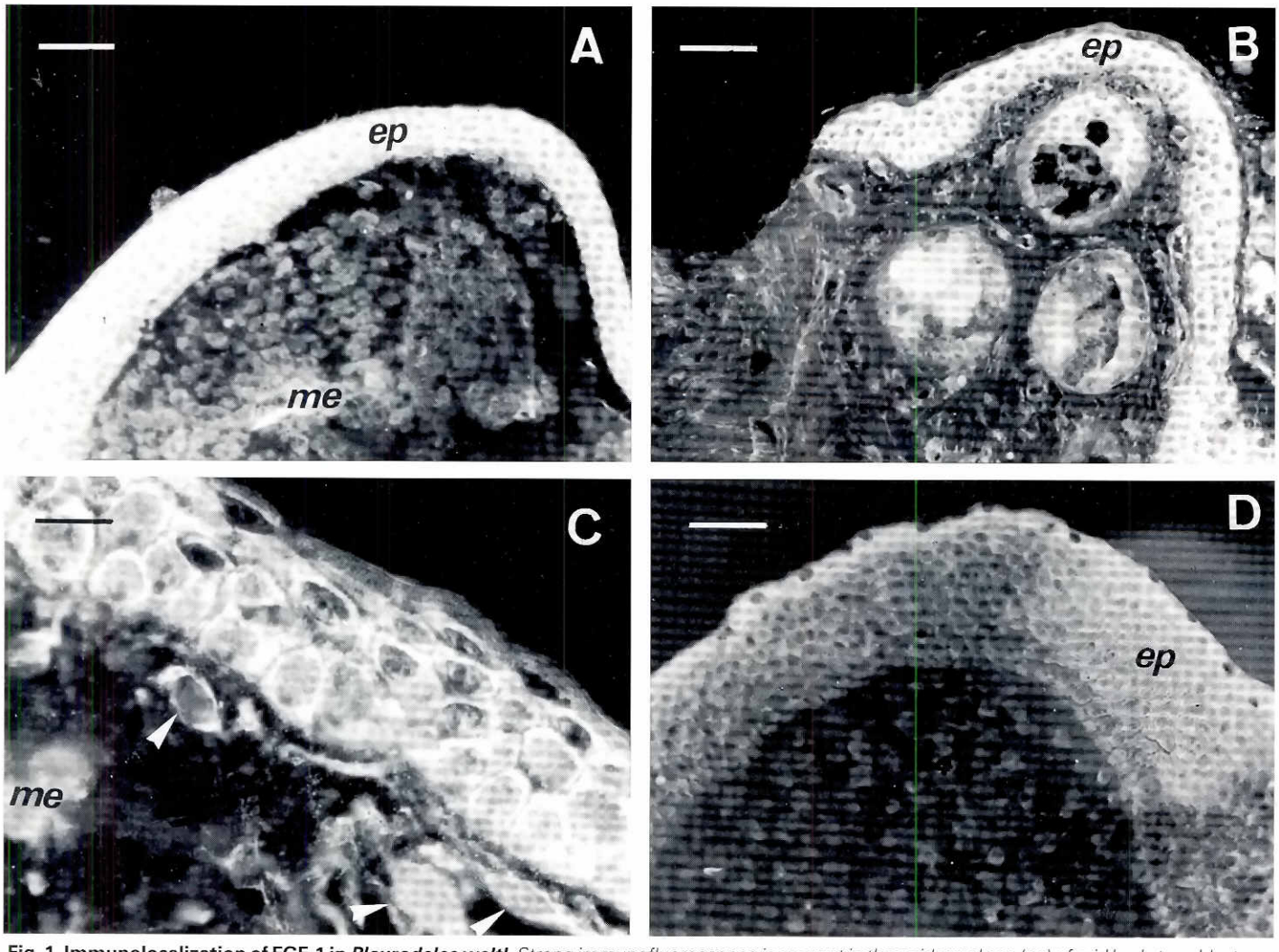

Fig. 1. Immunolocalization of FGF-1 in *Pleurodeles waltl*. *Strong immunofluorescence is present in the epidermal cap (ep) of mid bud stage blastema* **(A)** *and the epidermis of the stump* **(B)**. *Immunoreactivity is localized both in the cytoplasm of the epidermal cells and cell periphery* **(C)**. *The cytoplasm of mesenchymal cells is weakly labeled with the FGF-1 antibody (arrow). No reactivity was observed with non-immune rabbit serum* **(D)**. *Bars: 100 μm for A,B,D; 50 μm for C.*

FGF-1 and FGF-2 stimulate the growth of many cell types but also have neurotrophic and angiogenic activities; all these activities which are of importance during wound healing and tissue repair (Greenhalg *et al.*, 1990) make FGF a potent molecule for limb regeneration. It was first shown that FGF is able to stimulate cell proliferation of denervated blastemas both *in vivo* (Mescher et Gospodarowicz, 1979; Chew and Cameron, 1983) as well as *in vitro* (Rathbone *et al.*, 1979; Carlone *et al.*, 1981; Merscher and Loh, 1981; Albert *et al.*, 1987; Boilly and Albert, 1988). Then the presence of FGF-1 in blastemas was demonstrated using heparin-sepharose chromatography and immunochemical methods (Boilly *et al.*, 1991). Moreover, specific low and high affinity FGF binding sites were characterized using iodinated FGF binding (Boilly *et al.*, 1991; Hondermarck and Boilly, 1992). Finally, expression of FGF receptors in limb blastemas was reported using *in situ* hybridization (Poulin *et al.*, 1993) or Northern blot analysis of blastema RNAs (Boilly *et al.*, 1995). Thus, these results clearly show that blastema cells are responsive to FGF but they do not demonstrate that this factor is actually involved in the regeneration process. In the present study, we have first of all confirmed the presence of FGF-1 in limb blastemas of the amphibian *Pleurodeles waltl* using immunocytochemistry. We then provide evidence *in vivo* that FGF controls blastema cell proliferation by using different reagents which interfere with FGF activity. There are several widely used chemicals which have been shown to bind, and therefore inhibit, activity of FGFs. In this study, we have used sulfated polysaccharides which bind to the heparin-binding domain of the growth factors and thus prevent their attachment to and activation of the high-affinity receptors. We have also used the anticancer drug suramin, which also inhibits the binding of growth factors to their receptors, FGF-saporin, specifically designed to kill FGF-responsive cells, and neutralizing FGF-1 antibody which specifically blocks the biological activity of FGF-1.

Results

Immunoreactive FGF-1 is present in limb blastemas

A very strong FGF-1 immunoreactivity was seen over the entire epidermis of the regenerate, especially in the epidermal cap of the blastema (Fig. 1A,B). This immunoreactivity seems specific since

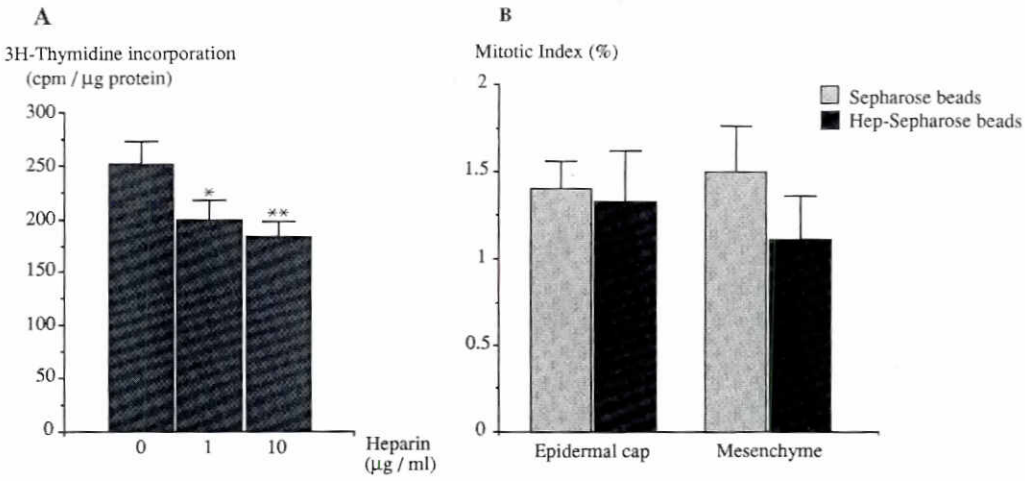

Fig. 2. Heparin effect on blastema cell proliferation in mid-bud blastema of *Pleurodeles waltl* 48 h after treatment. **(A)** *^{3}H thymidine incorporation (cpm/µg protein) after injection with 5 µl of PBS (control) or of heparin (1 and 10 µg/ml).* **(B)** *Mitotic index (%) in epidermal cap and mesenchyme after treatment with sepharose beads or heparin-sepharose beads (hep-sepharose beads). *Significantly lower (P<0.05) than control. **Significantly lower (P<0.01) than control.*

it does not appear in the absence of FGF-1 antibody or when FGF-1 is replaced by a rabbit non-immune serum (Fig. 1D). The antigen seems to be localized in the cytoplasm of epidermal cells and/or the cell periphery. The basement membrane, when present, was strongly labeled (not shown). In contrast to the epidermal cap, the blastema mesenchyme does not display strong FGF-1 labeling due to the fact that mesenchymal cells are dispersed; nevertheless, when observed at high magnification, the cytoplasm of mesenchymal cells appears weakly labeled with the FGF-1 antibody (Fig. 1C).

Sulfated polysaccharides decrease cell proliferation in limb blastemas

Whole blastemas treated with 1 µg/ml and 10 µg/ml heparin showed a significant decrease of 21% and 27% ^{3}H thymidine incorporation, respectively (Fig. 2). No hemorrhagic effect was observed since mid-bud blastemas of *Pleurodeles* are not yet well-vascularized. A decrease was also observed in the mitotic index with other polysaccharides (Fig. 3). Pentosan polysulfate (5 µg/ml) induced a 29% and 30% mitotic index decrease in the mesenchyme and epidermal cap, respectively (Fig. 3). With carrageenan, we observed a significant decrease (40 and 45%) only in the mesenchyme for a 10 µg/ml and a 50 µg/ml dose, respectively (Fig. 3). Conversely, when we used an immobilized heparin (bound to sepharose beads), the mitotic index did not decrease significantly (Fig. 2).

Suramin decreases cell proliferation in limb blastemas

Suramin, a drug which inhibits the binding of a variety of growth factors to their cell surface receptors, decreased the mitotic index of both mesenchymal and epidermal cells of limb blastemas. This effect was dose-dependent and similar for these two cell types; a non-toxic dose of 300 µg/ml reduced cell proliferation by 48% and 64% in mesenchyme and epidermal cap, respectively (Fig. 4).

FGF-saporin reduces cell proliferation in limb blastemas

FGF-saporin (1.3 ng/µl) induced a 37% significant decrease of ^{3}H thymidine incorporation, while saporin alone (0.75 ng/µl) had no effect (Fig. 5). At the mitotic index level, we observed a significant decrease (45%) in the mesenchyme while the decrease of mitotic index of the epidermal cap was not significant (20%) (Fig. 5).

Neutralizing anti FGF-1 decreases ^{3}H thymidine incorporation in limb blastemas

In the presence of neutralizing anti FGF-1 (3D12), ^{3}H thymidine incorporation in whole blastemas decreased by 44% compared to blastemas which received an equivalent quantity of PBS. The mitotic index also decreased, both in the mesenchyme and in the epidermal cap (45% and 32% respectively) with this antibody (Fig. 6). On the other hand, the injection of the same quantity on the non-relevant antibody 13F4 did not alter either ^{3}H thymidine incorporation or mitotic index (Fig. 6).

Discussion

Although they lack a peptide secretory signal, FGF-1 and FGF-2 are widely distributed in the EC compartment. Because of their

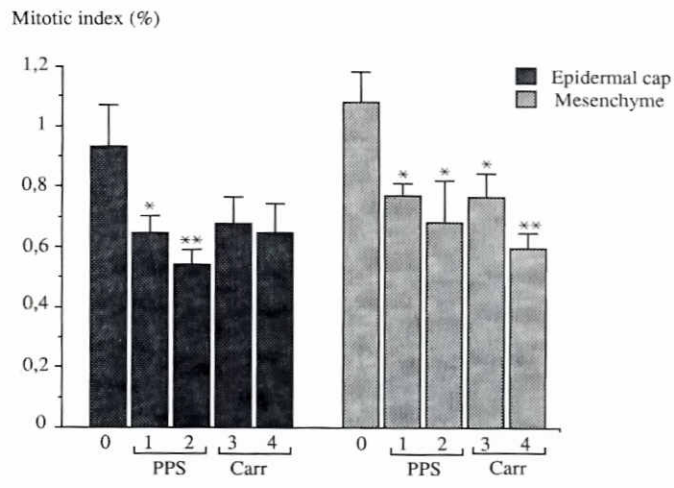

Fig. 3. Sulfated polysaccharide effect on cell proliferation of mid-bud stage blastema of *Pleurodeles waltl* 48 h after treatment. *Mitotic index (%) in epidermal cap and mesenchyme after treatment with 2 agarose beads soaked with PBS* **(0)** *or pentosan polysulfate (PPS), 5 µg/ml* **(1)** *and 10 µg/ml* **(2)** *or ι-carrageenans (Carr), 10 µg/ml* **(3)** *and 50 µg/ml* **(4)**. **Significantly lower (P< 0.05) than control. **Significantly lower (P<0.01) than control.*

strong heparin affinity, they are present in the ECM and especially in the basement membrane. However, this does not necessarily mean that these factors are active; they appear only to be stored in the heparan sulfate component of ECM. That is why they are sometimes considered as "wound hormones" ready to work when necessary, i.e. in case of injury (Ku and D'Amore, 1995). The high level of FGF-1 we extracted from the epidermal cap of axolotl limb blastemas (Boilly *et al.*, 1991) is therefore not surprising since this tissue contains a large amount of heparan sulfate (Boilly *et al.*, 1995).

In *Pleurodeles waltl*, using immunocytochemistry, we also observed that FGF-1 is mainly present in the epidermal cap; the same observation was made earlier on axolotls (Boilly, 1989). In *Pleurodeles*, immunolocalization of FGF-1 concerns both the cytoplasm of epidermal cells as well as cell periphery. The presence of intracellular FGF-1 filling the whole perinuclear space indicates that this factor is probably produced in these cells. The function of keratinocytes as FGF-producing cells is already known since Halaban*et al.* (1988) demonstrated that these cells produce FGF-2 which stimulates the underlying melanocytes, an effect we also observed in neural plate-derived cells from *Pleurodeles* (Maufroid *et al.*, 1996). The presence of FGF-1 around epidermal cell may be a storage form of this growth factor. How it is released in order to stimulate mesenchymal cells and/or epidermal cells represents the major problem of the function of FGF during limb regeneration. Whatever the mechanism of FGF-1 release from the epidermal cap, we propose that the bioavailability of FGF in mesenchyme controls blastema growth. Thus, polysaccharide sulfates such as heparin, carrageenan and pentosan polysulfate are able to decrease both ^{3}H thymidine incorporation and the mitotic index in blastemas treated by these compounds as previously shown in other cells such as cancer cells (Coombe *et al.*, 1987; Wellstein *et al.*, 1991; Hoffman, 1993; Hoffman and Sykes, 1993; Swain *et al.*, 1993; Kilfeather *et al.*, 1995). The present results are in agreement with many *in vitro* assays and observations *in vivo* concerning the relation between proliferative activity

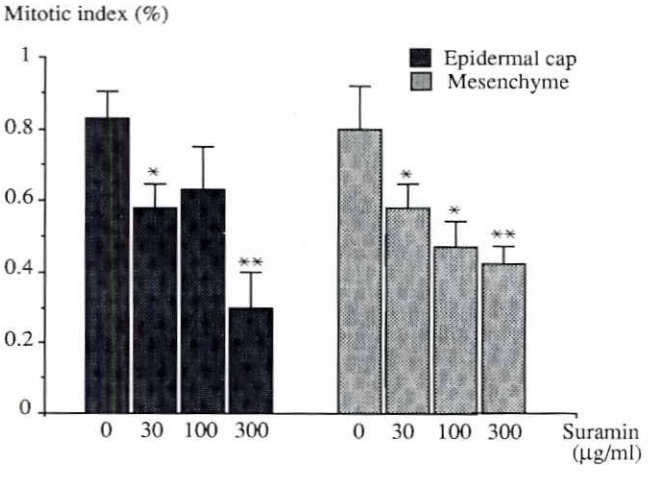

Fig. 4. Suramin effect on cell proliferation of mid bud stage blastema of *Pleurodeles waltl* 48 h after treatment. *Mitotic index (%) in epidermal cap and mesenchyme with 2 agarose beads soaked with PBS (control) or suramin (30, 100 and 300 µg/ml). *Significantly lower (P<0.05) than control. **Significantly lower (P<0.01) than control.*

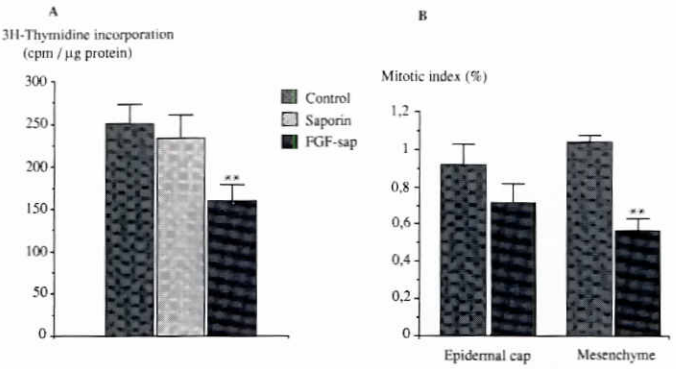

Fig. 5. FGF-saporin and saporin effect on blastema cell proliferation in mid-bud blastema of *Pleurodeles waltl* 48 h after treatment. (A) *^{3}H thymidine incorporation (cpm/µg protein) after injection with 5 µl of PBS (control), of saporin (0.75 ng/µl) and of FGF-saporin (1.3 ng/µl).* **(B)** *Mitotic index (%) in epidermal cap and mesenchyme after treatment with 2 agarose beads soaked with PBS (control) or FGF-saporin (1.3 ng/µl) (2). **Significantly lower (P<0.01) than control.*

and heparan sulfate level in different developmental models (Hondermarck *et al.*, 1992a,b) including limb regeneration (Boilly *et al.*, 1995). The latter study demonstrated a good correlation between the decrease of mitotic activity in denervated blastemas and the increase of HS synthesis in the mesenchyme. Most certainly, we do not know if the polysaccharide sulfate trapped only endogenous FGFs because many other growth factors bind to heparin (Fernig and Gallagher, 1994), but we can assume that they control at least FGF induced growth. Some polysaccharide sulfates, such as pentosan polysulfate, affected the growth of both the mesenchyme and the epidermal cap; this result led us to think that the growth of the epidermal cap might also depend on heparin binding growth factors as is the case for mammalian keratinocytes, the proliferation of which is stimulated by different types of FGFs such as FGF-1 (Shipley *et al.*, 1989) and under the dependence of keratinocyte growth factor (FGF-7) from mesenchymal cells (Rubin *et al.*, 1995). Morphogenesis during limb development depends on reciprocal interactions between epidermal cap and mesenchyme (Tabin, 1995 for review); thus treatments which limit the growth of mesenchyme might influence indirectly the growth of the epidermal cap by limiting the release of some factors from mesenchymal cells. Interestingly, we did not observe an effect on epidermal cap with less diffusible sulfated polysaccharides like carrageenan which affect only the mesenchyme. In addition, when there is no diffusion of the sulfated polysaccharides (immobilized heparin), no significant effect on either mesenchyme or epidermal cap was observed.

The effects of polysaccharide sulfates on blastema growth cannot be attributed only to their inhibition of FGF activity because they also interact with other heparin-binding growth factors. Nevertheless, we were able to show that FGF influences blastema growth by experimentally targeting FGF receptors. The FGF-2 saporin complex is known to bind to FGF receptors and to kill cells bearing these receptors following the internalization of the FGF-saporin receptor complex (Lappi *et al.*, 1989; Ying *et al.*, 1994). After FGF-saporin treatment, we observed a clear decrease of mitotic index in mesenchyme while saporin alone did not influence cell growth. This result indicates that at least some mesenchymal

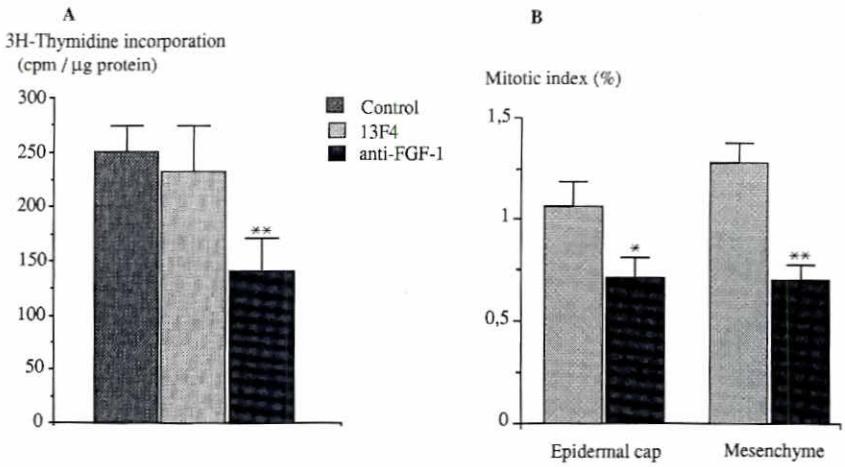

Fig. 6. Neutralizing FGF-1 antibody effect on blastema cell proliferation in mid-bud blastema of *Pleurodeles waltl* 48 h after treatment. **(A)** *^{3}H thymidine incorporation (cpm/µg protein) after injection with 5 µl of PBS (control), of a non-relevant antibody (13F4) or neutralizing anti FGF-1.* **(B)** *Mitotic index (%) in epidermal cap and mesenchyme after injection of a non-relevant antibody (13F4) or the neutralizing FGF-1 antibody. *Significantly lower (<0.05) than control. **Significantly lower (P<0.01) than control.*

cells possess FGF receptors, bind FGF-saporin and are consequently killed; this is in agreement with the expression of receptors for FGF-2 in blastema mesenchyme, namely FGFR-1 and the bek variant of FGFR-2 (Poulin *et al.* 1993; Poulin and Chiu, 1995). A similar decrease of mesenchymal cell growth was also obtained in suramin-treated blastemas. This agent, which disrupts the interaction of growth factors with their receptors, is known for its inhibitory action on protein kinase C in tumors (Hensey *et al.*, 1989; Myers *et al.*, 1992) and regeneration (Daller *et al.*, 1994); protein kinase C has also been implicated in amphibian limb regeneration (Boilly *et al.*, 1992; Martelly *et al.*, 1996). Its action on FGFs is well documented; thus proliferation (Leith *et al.*, 1992; Bernardini *et al.*, 1993) or neovascularization (Pesenti *et al.*, 1992), two classical effects of these growth factors, are prevented by suramin. It seems that the major effect of suramin is to cause microaggregation and, in some cases, conformational changes of the growth factor; interestingly, in the case of FGF-1, suramin interacts at or near its heparin binding site and induces aggregation of this growth factor to at least a hexameric state (Middaugh *et al.*, 1992).

The presence of FGF receptors on mesenchymal cells of limb blastemas is already known since cultured mesenchymal cells are responsive to FGF-1 and FGF-2 (Albert *et al.*, 1987) and since FGF receptors are expressed in these cells (Poulin *et al.*, 1993; Boilly *et al.*, 1995; Poulin and Chiu, 1995). Moreover, Scatchard analysis of FGF binding and FGF binding *in situ* (Boilly *et al.*, 1991; Hondermarck and Boilly, 1992) clearly showed that mesenchymal cells possess FGF receptors. Nevertheless, as FGF-2 binds to FGF receptors which are also recognized by FGF-1 (Partanen *et al.*, 1993), although the result we obtained with FGF-2-saporin clearly indicates that FGF is concerned with blastema cell growth, it does not distinguish which member of the FGF family is involved in blastema growth. Thus, the results we obtained with the neutralizing anti-FGF-1 (3D12), which was able to specifically reduce blastema growth by 44%, suggest that FGF-1 plays an important function in blastema growth. This is in agreement with earlier results which demonstrate that this growth factor can be extracted from both epidermal cap and mesenchyme while FGF-2 was not biochemically detected (Boilly *et al.*, 1991). It is possible that other members of the FGF family might be involved in limb regeneration as they are in limb development e.g., FGF-4 (Niswander *et al.*, 1993), FGF-8 (Heikinheimo *et al.*, 1994). Further investigations will be needed to clarify this point. It is also of great importance to know: 1) the source of FGF-1 in limb blastemas; 2) if it is produced by epidermal cells; 3) the conditions of its release; and 4) how the function of this growth factor is modulated during regeneration, especially by another important factor of limb regeneration i.e. regenerating nerves (Boilly and Bauduin, 1988).

Materials and Methods

Animals

We studied limb blastemas of *Pleurodeles waltl* from 6 months to 1 year-old. Blastemas were obtained after amputation under anesthesia (MS 222 Sandoz, 1‰) of both forelimbs. Amputated animals were maintained at 20±1°C. All experiments were conducted on mid-bud stage blastemas, a stage characterized by high proliferative activity and nerve dependence (Boilly *et al.*, 1986).

Chemicals and antibodies

Sulfated polysaccharides (heparin, ι-carrageenan, pentosan polysulfate) were from Sigma, Suramin (Germanin) from Bayer (Germany). Heparin-Sepharose and Sepharose beads were from Pharmacia (CL-6B). The FGF-saporin, a gift from D.A. Lappi (La Jolla, CA, USA), is a conjugate of FGF-2 and saporin-6 (a ribosome-inactivating protein isolated from *Saponaria officinalis*) in equimolar amounts (Lappi *et al.*, 1989).

FGF-1 polyclonal antibodies were a kind gift from Dr. Y. Cao (Stockholm, Sweden) and Dr. J. McAvoy (Sydney, Australia). FGF-1 monoclonal antibody 3D12 was provided by Dr. I. Hendry (Canberra, Australia). The non-relevant antibody used (13F4) was an avian early myogenic cell and differentiated muscle marker (Rong *et al.*, 1987), a gift from C. Ziller (Nogent s/ Marne, France).

Immunocytochemistry

Blastemas were fixed either in methanol (-20°C) or in 4% paraformaldehyde (Merck) (PBS 0.15 M NaCl, pH 7.2), washed in 5% sucrose in PBS and processed for inclusion in polyethylene glycol-400 distearate (Aldrich) (Norenburg and Barrett, 1987).

Immunolocalization of FGF-1 was performed on 8 to 10 µm sections with polyclonal antibodies and stained using either a FITC or a peroxydase labeled secondary antibody (anti-rabbit secondary antibodies were from Sigma).

Animal experiments

All chemicals introduced into the blastema were diluted in PBS, NaCl 0.15 M (pH 7.2). They were either directly injected into blastemas or diffused from Affi-gel blue beads (Biorad), previously soaked in the chemical solution and mechanically inserted into the blastemas. For injection, 5

µl of solution was slowly delivered to the blastema with a glass microsyringe. The contralateral blastema received the same volume of PBS and served as control. Affi-gel blue beads were soaked in solution for 24 h at 4°C and implanted under the epidermal cap (2 beads per blastema). The contralateral blastema received 2 beads soaked in PBS only.

Proliferative activity

Forty-eight hours after treatment with the different chemicals, the proliferative activity of blastemas was assayed either with ^{3}H thymidine incorporation in whole blastemas or by mitotic index evaluation; the latter method allowed for the differentiation of the effects of treatments on each of the blastemal compartments (epithelial or mesenchymal).

^{3}H-thymidine incorporation was measured after 24 h incubation (25°C) of freshly dissected blastemas in the presence of 3 µCi/ml of ^{3}H thymidine (ICN, specific activity 2 Ci/mmole) in Leibovitz medium (L15 Sigma, L 4386) diluted to 70% and complemented with insulin (0.15 U/ml) (Organon) and antibiotics (Penicillin/Streptomycin, Eurobio) as previously described (Lassalle, 1983). Thymidine incorporation is indicated in relation to total blastema protein (Bradford's method, 1976). Six blastemas were used for each type of experiment.

For mitotic index estimation, blastemas were fixed (Bouin Hollande fixative) and processed for paraffin embedding. Mitotic figures (metaphases to telophases) were counted on 7 µm sections and the mitotic index estimated from at least 10,000 cells per blastema. The mitotic index was recorded in each of the two parts of the blastema, the epidermal cap and the underlying mesenchyme. Three blastemas were used for each type of experiment. Statistical analysis were performed using the Student's *t* test.

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References

ALBERT, P., BOILLY, B., COURTY, J. and BARRITAULT, D. (1987). Stimulation in cell culture of mesenchymal cells of newt limb blastema by EDGF I or II (basic or acidic FGF). *Cell Differ. 21*: 63-68.

BERNARDINI, N., GIANNESSI, F., BIANCHI, F., DOLFI, A., LUPETTI, M., CITTI, L., DANESI, R. and DEL TACCA, M. (1993). Involvement of basic fibroblast growth factor in suramin-induced inhibition of V79/AP4 fibroblast cell proliferation. *Brit. J. Cancer 67*: 1209-1215.

BOILLY, B. (1989). Production of growth factors by the blastema during limb regeneration of urodeles (Amphibia). In *Recent Trends in Regeneration Research* (Eds. V. Kiortsis, S. Koussoulakos and H. Wallace). Plenum Press, New York, pp. 81-96.

BOILLY, B. and ALBERT, P. (1988). Control of blastema cells proliferation during newt limb regeneration. *In vitro* cell culture study. In *Control of Cell Proliferation and Differentiation during Regeneration* (Ed. H.J. Anton). Monogr. Dev. Biol., Vol. 21. Karger, Basel, pp. 1-8.

BOILLY, B. and BAUDUIN, B. (1988). Production *in vitro* by spinal cord of growth factor(s) acting on newt limb regeneration: influence of regenerating nerve fibers. *Dev. Brain Res. 38:* 155-160.

BOILLY, B., CAVANAUGH, K.P., HONDERMARCK, H., BRYANT, S.V. and BRADSHAW, R.A. (1991). Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotl and binds specifically to blastema tissues. *Dev. Biol. 145*: 302-310.

BOILLY, B., LHEUREUX, E., BOILLY-MARER, Y. and BART, A. (1990). Cell interactions and regeneration control. *Int. J. Dev. Biol. 34:* 219-231.

BOILLY, B., MOUSTAFA, Y. and OUDKHIR, M. (1986). Image analysis of blastema cell proliferation in the denervated limb regenerate of the newt. *Differentiation 32:* 208-214.

BOILLY, B., OUDGHIR, M., DEUDON, E., BOILLY-MARER, Y. and HONDERMARCK, H. (1995). Nerve dependent sulphated glycosaminoglycan synthesis in limb regeneration of the newt *Pleurodeles* waltl. *Roux Arch. Dev. Biol. 204*: 509-512.

BOILLY, B., OUDKHIR, M., MORACZEWSKI, G. and MARTELLY, I. (1992). Signal transduction pathway of nerve-derived mitogen in regenerating limb of newt. In *Keys for Regeneration* (Eds. CH. Taban and B. Boilly). Monogr. Dev. Biol. Vol. 23. Basel, Karger, pp. 93-99.

BOILLY, B., SHI, D.L., DESBIENS, X., LHEUREUX, E. and BOUCAUT, J.C. (1995). Expression of FGF receptors during limb regeneration. *Proc. Aust. Neurosci. Soc. 6:* 243.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem. 72:* 248-254.

CARLONE, R.L., GANAGARAJAH, M. and RATHBONE, M.P. (1981). Bovine pituitary Fibroblast Growth Factor has neurotrophic activity on newt limb regenerates and skeletal muscles *in vitro*. *Exp. Cell Res. 132:* 15-21.

CHERNOFF, E.A.G. and STOCUM, D.L. (1995). Developmental aspects of spinal cord and limb regeneration. *Dev. Growth Differ. 37:* 133-147.

CHEW, K.E. and CAMEROUN, J.A. (1983). Increase in mitotic activity of regenerating Axolotl limb by growth factor-impregnated implants. *J. Exp. Zool. 226:* 325-329.

COOMBE, D.R., PARISH, C.R., RAMSHAW, I.A. and SNOWDEN, J.M. (1987). Analysis of the inhibition of tumor metastasis by sulphated polysaccharides. *Int. J. Cancer 39:* 82-88.

DALLER, J.A., BUKLEY, A.R., VAN HOOK, F.W., BUKLEY, D.J. and PUTNAM, C.W. (1994). Suramin, a protein kinase C inhibitor, impairs hepatic regeneration. *Cell Growth Differ. 5:* 761-767.

FABER, J. (1971). Vertebrate limb ontogeny and limb regeneration: morphogenetic parallels. In *Advances in Morphogenesis*, Vol. 9. Academic press, Inc., New York and London, pp. 127-147.

FERNIG, D.G. and GALLAGHER, J.T. (1994). Fibroblast growth factor and their receptors: an information network controlling tissue growth, morphogenesis and repair. *Prog. Growth Factor Res. 5:* 353-377.

GLOBUS, M. and VETHAMANY-GLOBUS, S. (1985). *In vitro* studies of controlling factors in newt limb regeneration. In *Regulation of Vertebrate Limb Regeneration* (Ed. R.E. Sicard). Oxford University Press, New York, pp. 106-127.

GOSPODAROWICZ, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. *J. Biol. Chem. 250*: 2515-2520.

GOSPODAROWICZ, D. and MESHER, A.L. (1980). Fibroblast growth factor and the control of vertebrate regeneration and repair. *Ann. NY Acad. Sci. 339:* 151-174.

GREENHALG, D.G., SPRUGEL, K.H., MURRAY, M.J. and ROSS, R. (1990). PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol. 136:* 1235-1246.

HALABAN, R., LANGDON, R., BIRCHALL, N., CUONO, C., BAIRD, A., SCOTT, G., MOELLMANN, G. and GUIRE, J.M. (1988). Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. *J. Cell Biol. 107:* 1611-1619.

HEIKINHEIMO, M., LAWSHE, A., SHACKLEFORD, G.M., WILSON, D.B. and MacARTHUR, C.A. (1994). Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limb and central nervous system. *Mech. Dev. 48:* 129-138.

HENSEY, C.E., BOSCOBOINIK, D. and AZZI, A. (1989). Suramin, an anti-cancer drug, inhibits protein kinase C and induces differentiation in neuroblastoma cell clone NB2A. *FEBS Lett. 258:* 156-158.

HOFFMAN, R. (1993). Carrageenans inhibit growth factor binding. *Biochem. J. 289*: 331-334.

HOFFMAN, R. and SYKES, D. (1993). Inhibition of binding of basic fibroblast growth factor to low and high affinity receptors by carrageenans. *Biochem. Pharmacol. 45:* 2348-2351.

HONDERMARCK, H. and BOILLY, B. (1992). Characterisation of fibroblast growth factor binding in regenerating limb blastemas of axolotl. In *Keys for Regeneration* (Eds. C.H. Taban and B. Boilly). Monogr. Dev. Biol. Vol. 23. Karger, Basel, pp. 110-115.

HONDERMARCK, H., COURTY, J., DAUCHEL, M.C., BARRITAULT, D. and BOILLY, B. (1992a). High and low affinity membrane binding sites for fibroblast growth factors in the developing chick brain. *Neurosci. Lett. 134*: 247-252.

HONDERMARCK, H., DEUDON, E. and BOILLY, B. (1992b). Embryonic brain-derived heparan sulfate inhibits cellular membrane binding and biological activity of basic fibroblast growth factor. *Dev. Brain Res. 68:* 247-253.

KILFEATHER, S.A., TAGOE, S., PEREZ, A.C., OKONA-MENSA, P.K., MATIN, R.

and PAGE, C.P. (1995). Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans. *Brit J. Pharmacol. 114*: 1442-1446.

KU, P.T. and D'AMORE, P.A. (1995). Regulation of basic fibroblast growth factor (bFGF) gene and protein expression following its release from sublethally injured endothelial cells. *J. Cell. Biochem. 58:* 328-343.

LAPPI, D.A., MARINEAU, D. and BAIRD, A. (1989). Biological and chemical characterization of basic FGF-saporin mitotoxin. *Biochem. Biophys. Res. Commun. 160:* 917-923.

LASSALLE, B. (1983). Synthèse d'ADN dans les blastèmes de régénération de *Pleurodeles waltlii* (Amphibien urodèle) en culture *in vitro*. Conditions de culture. *Biol. Cell. 47*: 187-192.

LEITH, J.T., PAPA, G., QUARANTO, L. and MICKELSON, S. (1992). Modification of the volumetric growth responses and steady-state hypoxic fractions of xenografted DLD-2 human colon carcinoma by administration of basic fibroblast growth factor or suramin. *Brit. J. Cancer. 66:* 345-348.

MARTELLY, I., OUDGHIR, M., MORACZEWSKI, J. and BOILLY, B. (1996). Activation of protein kinase C during newt limb regeneration: effect of denervation. *Biochem. Cell Biol.* (In press).

MAUFROID, J.P., BRADSHAW, R.A., BOILLY, B. and HONDERMARCK, H. (1996). Nerve growth factor induced neurite outgrowth from amphibian neuroepithelial precursor cells is prevented by dipeptides inhibiting ubiquitin-mediated proteolysis. *Int. J. Dev. Biol. 40:* 609-611.

MESCHER, A.L. and GOSPODAROWICZ, D. (1979). Mitogenic effect of a growth factor derived from myelin on denervated regenerates of newt forelimbs. *J. Exp. Zool. 207:* 497-503.

MESCHER, A.L. and LOH, J.J. (1981) Newt forelimb regeneration blastemas *in vitro*: cellular response to explantation and effects of various growth-promoting substances. *J. Exp. Zool. 216:* 235-245.

MESCHER, A.L. and MUNAIM, S.I. (1984). "Trophic" effect of transferrin on amphibian limb regeneration blastemas. *J. Exp. Zool. 230:* 485-490.

MIDDAUGH, C.R., MACH, H., BURKE, C.J., VOLKIN, D.B., DABORA, J.M., TSAI, P.K., BRUNER, M.W., RYAN, J.A. and MARFIA, K.E. (1992). Nature of the interaction of growth factor with suramin. *Biochemistry 31:* 9016-9024.

MYERS, C., COOPER, M., STEIN, C., LA ROCCA, R., WALTHER, Mc.C., WEISS, G., CHOYKE, P., DAWSON, N., STEINBERG, S., UHRICH, M.M., CASSIDY, J., KOHLER, D.R., TREPEL, J. and LINEHAN, M. (1992). Suramin: a novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer. *J. Clin. Oncol. 10:* 881-889.

NISWANDER, L., TICKLE, C., VOGEL, A., BOOTH, I. and MARTIN, G.R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell 75:* 579-587.

NORENBURG, J. and BARRET, J. M. (1987). Steedman's polyester wax embedment and de-embedment for light and scanning electron microscopy. *J. Electron Microsc. Tech. 6:* 35-41.

PARTANEN, J., VAINIKKA, S., ALITALO, K. (1993). Structural and functional specificity of FGF receptors. *Philos. Trans. R. Soc. Lond. 340:* 297-303.

PESENTI, E., SOLA, F., MONGELLI, N., GRANDI, M. and SPREAFICO, F. (1992). Suramin prevent neovascularisation and tumor growth though blocking of basic fibroblast growth factor activity. *Brit. J. Cancer 66:* 367-372.

POULIN, M.L. and CHIU, I.M. (1995). Re-programming of expression of the KGFR and bek variants of fibroblast growth factor receptor 2 during limb regeneration in newts *(Notophthalmus viridescens). Dev. Dynamics 202:* 378-387.

POULIN, M.L., PATRIE, K.M., BOTELHO, M.J., TASSAVA, R.A. and CHIU, I.M. (1993). Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts *(Notophthalmus viridescens). Development 119:* 353-361.

RATHBONE, M.P., CARLONE, R.L. and GANAGARAJAH, M. (1979). Neurotrophic like effects of bovine pituitary fibroblast growth factor on regenerating limb blastema and skeletal muscles of newt *in vitro. Am. Zool. 19:* 925.

RONG, P.M., ZILLER, C., PENA-MELIAN, A. and LE DOUARIN, N.M. (1987). A monoclonal antibody specific for avian early myogenic cells and differentiated muscle. *Dev. Biol. 122:* 338-353.

RUBIN, J.S, BOTTARO, D.P., CHEDID, M., MIKI, T., RON, D., CHUNHA, G.R. and FINCH, P.W. (1995). Keratinocytes growth factor as a cytokine that mediates mesenchymal-epithelial interaction. In *Epithelial Mesenchymal Interactions in Cancer*, Vol. 74 (Eds. I.D. Goldberg and E.M. Rosen). Birkhauser Verlag, Basel, pp. 69-87.

SHIPLEY, G.D., KEEBLE, W.W., HENDRICKSON, J.E., COFFEY, R.J. and PITTELKOW, M.R. (1989). Growth of normal human keratinocytes and fibroblast in serum-free medium is stimulated by acidic and basic fibroblast growth factor. *J. Cell. Physiol. 138:* 511-518.

SMITH, M.J., GLOBUS, M. and VETHAMANY-GLOBUS, S. (1995). Nerve extracts and substance P activate the phosphatidylinositol signaling pathway and mitogenesis in newt forelimb regenerates. *Dev. Biol. 167*: 239-251.

SWAIN, S., WELLSTEIN, A., PARKER, B., LIPPMAN, M., STEAKLEY, C. and DeLAP, R. (1993). Heparin-binding growth factor blockade with pentosan polysulfate. *Ann. NY Acad. Sci. 698:* 63-70.

TABIN, C. (1995). The initiation of the limb bud: growth factors, hox genes, and retinoids. *Cell 80:* 671-674.

TSONIS, P.A. (1996). *Limb Regeneration.* Cambridge University Press, Cambridge.

WALLACE, H. (1981). *Vertebrate Limb Regeneration.* J. Wiley and Sons Ltd., New York.

WELLSTEIN, A., ZUGMAIER, G., CALIFANO, J.A. III, KERN, F., PAIK, S. and LIPPMAN, M.E. (1991). Tumor growth dependent on kaposi's sarcoma-derived fibroblast growth factor inhibited by pentosan polysulfate. *J. Natl. Cancer Inst. 83:* 716-720.

YING, W., MARTINEAU, D., BEITZ, J., LAPPI, D.A. and BAIRD, A. (1994). Anti-B16-F10 melanoma activity of a basic fibroblast growth factor-saporin mitotoxin. *Cancer 74:* 848-853.

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