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Authors

Boilly, B
Cavanaugh, KP
Thomas, D
[et al.](#)

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Acidic Fibroblast Growth Factor Is Present in Regenerating Limb Blastemas of Axolotls and Binds Specifically to Blastema Tissues

BÉNONI BOILLY,^{*1} KATHLEEN P. CAVANAUGH,² DIDIER THOMAS,^{*} HUBERT HONDERMARCK,
* SUSAN V. BRYANT,[†] AND RALPH A. BRADSHAW[‡]

^{*}Laboratoire de Biologie des Facteurs de Croissance, Université de Lille 59655 Villeneuve d'Ascq Cedex France; [†]Developmental Biology Center and [‡]Department of Biological Chemistry, University of California, Irvine California 92717

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The growth of regenerating limbs of amphibians depends upon proliferation of the blastema cells that accumulate beneath the epidermal cap. The epidermal cap is known to be mitogenic for the blastema cells. We have extracted a mitogenic activity from both the mesenchymal and epidermal (epidermal cap) components of cone stage blastemas which is retained on heparin-Sepharose and elutes with 1.15 M NaCl. This fraction stimulates neurite outgrowth of PC12 cells and [³H]thymidine incorporation into CCL 39 cells and is potentiated by heparin. The 2 M fraction was inactive. The heparin-Sepharose-purified growth factor cross-reacts with bovine acidic FGF polyclonal antibodies and shows a M_r of 16,000 on Western blots. Blastema membranes contain specific high affinity binding sites ($K_d = 25$ pM; capacity = 30 fmole/mg protein) and low affinity binding sites ($K_d = 18$ nM; capacity = 30 pmole/mg protein) for aFGF as revealed by Scatchard analysis. [¹²⁵I]-aFGF which is bound specifically by both the epidermal cap and mesenchyme of blastema frozen sections is displaced by an excess of unlabeled factor and inhibited by heparin. Heparinase treatment and 2 M NaCl washing which decreased the binding was fourfold more efficient for epidermal cap than for mesenchyme suggesting the presence of high affinity receptors in the latter tissue. The presence of aFGF (or a closely related molecule) in blastemas is consistent with our earlier results that showed stimulation of proliferation of cultured blastema cells by acidic or basic FGF or heparin alone. These results suggest the possibility that aFGF is stored in the epidermal cap during limb regeneration and that it stimulates the proliferation of the underlying mesenchyme. © 1991 Academic Press, Inc.

INTRODUCTION

Limb regeneration in amphibians results from the rapid proliferation of blastema cells followed by differentiation. Cell proliferation is under the control of diffusible factors mainly derived from transected nerves which produce the so-called NTF ("neurotrophic factor"), the nature of which is still unknown (Singer, 1974, 1978). It has been suggested that NTF could be FGF³ because this growth factor stimulates the mitotic index of denervated blastemas after *in vivo* injection (Mescher and Gospodarowicz, 1979; Gospodarowicz and Mescher, 1980) and a stimulatory effect of FGF on cultured blas-

temas (Carlone *et al.*, 1981; Mescher and Loh, 1981; Mescher, 1983) or cultured blastema cells (Albert *et al.*, 1987) has been reported.

The epidermal cap of blastemas also plays an important role in blastema cell proliferation (Stocum, 1985; Globus and Vethamany-Globus, 1985). Hence regeneration is halted after removal (Thornton, 1957) or X-irradiation (Lheureux and Carey, 1988) of the epidermal cap or by insertion of skinless stumps or blastemas into muscles (Polezhaev and Faworina, 1935), the body cavity (Goss, 1956), or the connective tissue of the dorsal fin (Stocum and Dearlove, 1972). In a similar manner, grafting a skin flap over the amputation surface, which impedes the formation of an epidermal cap, also prevents limb regeneration (Polezhaev and Faworina, 1935; Mescher, 1976). On the other hand, extracts from whole limb blastemas show some stimulatory effect on denervated (Deck and Futch, 1969; Burnett *et al.*, 1971) or X-irradiated limbs (Deck and Dent, 1970). With a sensitive bioassay using cultured blastema cells (Albert and Boilly, 1986) we recently demonstrated the presence of potent mitogenic activity in the epidermal cap (Boilly and Albert, 1990); we also showed that extracts from blastemal mesenchyme stimulate blastema cell proliferation (Boilly and Albert, 1990). Although these extracts

¹ To whom correspondence and reprint requests should be addressed.

² Present address: Cortex Pharmaceuticals, 15241 Barranca Parkway, Irvine, CA 92718.

³ Abbreviations used: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CCL39, Chinese hamster fibroblasts ATCC cell line; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; MEM, minimum essential medium; PC12, rat pheochromocytoma cell line; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline 0.15 M NaCl.

were less mitogenic than those from epidermal cap, they were more potent for blastema cells than nerve extracts (Albert and Boilly, 1986; Boilly and Albert, 1988).

The sensitivity of blastema cells to FGF (Albert *et al.*, 1987) together with the high mitogenic activity of blastema extracts (Boilly and Albert, 1990) prompted us to look for the presence of FGF in regenerating blastemas. We report here that both epidermal cap and mesenchyme of regenerating axolotl limbs contain aFGF and that these tissues specifically bind this growth factor.

MATERIAL AND METHODS

Limb blastemas were obtained after amputation of both forelimbs in *Ambystoma mexicanum* of 6 months to one year old. The blastemas were collected at the mid-bud stage and some were further dissected to separate the epidermal cap from the mesenchyme. All of the samples (whole blastemas, epidermal cap, mesenchyme) were immediately frozen in liquid nitrogen and stored at -70°C before use.

Extraction of FGF-like Material

Five sets of blastema tissues (110 whole blastemas, 0.5 g; 180 whole blastemas, 1.2 g; 950 whole blastemas, 5.0 g; 360 epidermal caps, 1.6 g; 360 blastemal mesenchyme, 1.0 g) were homogenized (Potter homogenizer) either in distilled water or in 10 mM Tris-HCl, pH 7.2/1 M NaCl (1/6 w/v) with or without proteases inhibitors (1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 5 kallikrein inhibitor units/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was sonicated and centrifuged (43,000g for 1 hr, 4°C) and the supernatant, adjusted to 0.3 M NaCl with 10 mM Tris-HCl, pH 7.2, was applied to a heparin-Sepharose (6B Pharmacia) column preequilibrated at room temperature in 10 mM Tris-HCl, pH 7.2/0.6 M NaCl. After application of the sample provided by the smallest set of blastemas (0.5 g of fresh tissue) the column was washed with the same buffer and eluted in one step with 8 ml of 10 mM Tris-HCl, pH 7.2/2 M NaCl. For the other sets of blastemas (180 and 950 whole blastemas, 360 epidermal caps, 360 blastemal mesenchymes) the column was sequentially eluted with the same buffer containing 0.6, 1.15, 2.0, or 3.0 M NaCl (8 ml for each step). The eluates were concentrated on Centricon 10 (Amicon) microconcentrators (10,000 cut-off). In order to avoid high salt concentration in the biological assays, some 2 and 3 M eluates were diluted and concentrated until a final concentration of 1.15 M in about the same volume as the 0.6 and 1.15 M samples (about 500 μl from 2 ml of eluates) was obtained.

Assay of FGF-like Material

Cell culture assays. Assays were performed on rat pheochromocytoma cells (PC12) and Chinese hamster

fibroblasts (CCL39). These cells respond to FGF by either neurite outgrowth (PC12 cells) (Rydel and Greene, 1987) or DNA synthesis (CCL39 fibroblasts) (Courtney *et al.*, 1987).

PC12 cells. PC12 cells were cultured in DMEM (Irvine Scientific) supplemented with 10% fetal calf serum and 5% horse serum. They were seeded in 24-well plates (500 μl of medium/well) pretreated with rat tail collagen at 6000 cells/well and stimulated by each of the samples every day or every 2 days 24 hr after plating. In some cases the medium was changed just before adding the sample in order to avoid high salt concentration. NGF or bFGF (5 or 50 ng/ml) was tested in parallel in the presence of a volume of buffer equal to that of the eluates.

CCL39 cells. CCL 39 were grown in MEM (Earle's salts) (GIBCO) and 10% fetal calf serum at 40,000 cells/well in 24-well plates (500 μl of medium/well). Cells were arrested 72 hr later by changing the medium to serum-free conditions for 24 hr. Cells were then stimulated by one addition of sample (1.15 or 2 M) for 24 hr while [^3H]thymidine (sp act 1 Ci/mmol, CEA; 1 Ci = 37 GBq) was added (1 $\mu\text{Ci}/\text{well}$) during the last 4 hr. After two washes with PBS 0.15 M NaCl and a trichloroacetic acid treatment the cells were lysed with 0.5 ml of 0.1 N NaOH and the radioactivity was counted after mixing the lysate with the liquid scintillation cocktail (Ready Safe, Beckman) with a beta counter (LKB Wallac).

For the PC12 and CCL39 cell culture assays, 10 or 20 μl of sample/well were added to the culture medium with or without 2.5 μl (12.5 ng/well) heparin (Sigma). All the assays were performed in triplicate.

ELISA

For ELISA, the 1.15 and 2 M samples were coated to microtiter wells (Nunc) overnight at $+4^{\circ}\text{C}$. The wells were postcoated with bovine serum albumin (fraction V, Sigma) and incubated (2 hr at room temperature) with polyclonal antibodies to aFGF (RD systems), or to bFGF (RD systems). The bound antibodies were revealed with a peroxidase-conjugated anti-rabbit IgG (Pasteur Products). Peroxidase activity was determined with *o*-phenylenediamine (Sigma) that was measured in the microtiter well with a microplate reader (Dynatech).

Electrophoresis and Western Blot Analysis

SDS/PAGE of 1.15 and 2 M samples was performed in 15% acrylamide gels according to Laemmli (1970) and the gels were silver stained (BioRad). Subsequent to PAGE, the proteins were transferred to an Immobilon (Millipore) membrane, treated with antibody to aFGF, and then with ^{125}I -protein A (DuPont). The blots were

radioautographed on Agfa film for 4 days at -70°C between Cronex intensifying screens (DuPont).

Binding Assays

Binding assays were performed on blastema membrane preparations and blastema frozen sections with FGF prepared from bovine brain and radiolabeled by the chloramine T method with Na^{125}I (Oris, France) (sp act of ^{125}I -FGF:100,000 to 150,000 cpm/ng).

Binding on blastema membrane preparations. Membranes were obtained from 550 blastemas as follows: After homogenization of blastemas in 20 mM Hepes buffer, pH 7.4 (containing 5 mM EDTA, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 5 kallikrein inhibitor units/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.3 M sucrose), and centrifugation of the homogenate (1000g, 20 min, 4°C), the supernatant was collected and centrifuged again (40,000g, 20 min, 4°C) and the final pellet, containing membranes, was suspended in the same buffer with 3 M MgCl_2 (30 min, 4°C). The membranes were pelleted a second time (40,000g, 20 min, 4°C) and washed three times in the Hepes buffer without EDTA in the presence of 1 mM MgCl_2 , 1 mM CaCl_2 , and 0.1 M NaCl, and finally stored at -80°C before use. Protein concentration of the membrane preparations was measured according to the method of Bradford (1976) using BSA as a standard.

Binding assays were carried out with 23 pM of ^{125}I -aFGF after optimization of the binding conditions as previously described (Hondermarck *et al.*, 1990) (the equilibrium binding was obtained after 30 min at 4°C in presence of 30 μg of membrane protein). Competitive binding assays were performed using increasing quantity of unlabeled aFGF up to 23 nM. After a 40-min incubation, membranes were collected and their radioactivity was measured with a gamma counter (Wallac, LKB). Scatchard analysis was done with the Ligand fitting program (Munson and Rodbard, 1980).

Binding on blastema frozen sections. Blastemas were frozen with liquid nitrogen immediately after dissection or after fixation (0.1% paraformaldehyde in PBS for 2 hr at 4°C). They were positioned on a frozen metal block with OCT compound (Miles) for histological techniques. Sections of 10 μm were cut at -20°C in a cryostat (Reichert-Jung), collected on gelatin-coated glass slides and stored dessicated at -70° before use the same week. Sections were then treated as follows: They were first rinsed in PBS/BSA (0.5%), soaked in PBS containing 2 M MgCl_2 for 30 min at 4°C , and rinsed again in PBS/BSA before incubation with ^{125}I -a- or bFGF. Sections were incubated with 0.5 ng ^{125}I FGF in PBS/BSA for 1 hr at room temperature. After incubation, sections were washed six times with PBS/BSA and then dried at room

temperature. Slides were then dipped in photoemulsion K2 (Ilford) diluted in distilled water (v:v), exposed 2 weeks at 4°C , developed with Kodak D19 B, and fixed with sodium hyposulfite. The sections were stained after development with nuclear red, mounted in Xam, and analyzed with a Samba 200 (Thomson TITN) microscopic image processor for silver grain counting on autoradiograms. Epidermal cap and mesenchyme were analyzed separately on the same section.

Different treatments of the sections such as competition experiments or chemical or enzymatic treatments were performed on sections before or after incubation with ^{125}I -aFGF or ^{125}I -bFGF (four assays for each treatment).

Competition experiments. Unlabeled aFGF or bFGF was mixed with its respective iodinated forms (500 $\times$ or 1000 $\times$ the amount of labeled FGF) and applied on the section before exposition.

Chemical treatments. Sections were treated with 2 M NaCl or 0.1 ng/ml heparin (Sigma) just after incubation with the labeled FGF.

Enzymatic treatments. Samples were treated with 50 U/ml heparinase II or chondroitinase ABC (Sigma) for 30 min at room temperature (30 μl solution of enzyme on each section) just before FGF binding.

Statistical significance was determined using Student's *t* test for unpaired observations.

RESULTS

The presence of FGF material in limb blastemas was demonstrated initially by testing the extracted material *in vitro* on PC12 and CCL39 cells which respond to FGF by neurite outgrowth and thymidine incorporation, respectively. This material was characterized by ELISA and Western blot. To understand the function of this FGF material in blastemas, we performed binding assays with iodinated FGF both on blastema membrane preparations and blastema sections.

Effect of Blastema Extracts on Neurite Outgrowth of PC12 Cells

Three additions (10 μl of the first 2 ml) of the 2 M eluate stimulated 5 ± 1 to $7 \pm 3\%$ of the cells to grow neurites, a percentage which rose to 10% in the presence of heparin. Heparin stimulated $5 \pm 2\%$ of the cells with samples from the 3rd and 4th ml elution, which gave a negative response without heparin. Samples from the 0.6 M and desalted 2 or 3 M eluates from the 180 whole blastema extract did not elicit a PC12 cell response even after three additions of the eluate. On the other hand, two additions of the 1.15 M eluate stimulated $25 \pm 8\%$ of the PC12 cells to grow neurites in the presence of heparin; without heparin, only $5 \pm 2\%$ of the cells exhibited

FIG. 1. [³H]Thymidine incorporation (cpm/well $\pm$ SD) in arrested CCL 39 fibroblasts after addition to the culture well (500 μ l of culture medium) of 20 μ l of 10 mM Tris-HCl, pH 7.2/1.15 M NaCl in the absence or presence of 2.5 μ l (12.5 ng) heparin; 20 μ l of the 1.15 M NaCl eluate of blastema-heparin-binding-proteins in the absence or presence of 2.5 μ l (12.5 ng) heparin; 10 μ l of 10 mM Tris-HCl, pH 7.2/2 M NaCl in the absence of 2.5 μ l (12.5 ng) heparin; and 10 μ l of the 2 M NaCl eluate of blastema heparin binding proteins in the absence or presence of 2.5 μ l (12.5 ng) heparin.

neurites after two additions of this eluate. By comparison, 50 ng/ml of NGF stimulated $10 \pm 4\%$ of the cells. We obtained similar results with the eluates obtained either from epidermal caps or blastemal mesenchyme. Only the 1.15 M eluate stimulated PC12 cells to grow neurites; with heparin the 1.15 M fraction from epidermal caps stimulated $30 \pm 7\%$ of the cells, while $15 \pm 5\%$ grew neurites after treatment with the 1.15 M eluate derived from mesenchymal cells.

Clearly, after heparin-Sepharose chromatography, only the 1.15 M eluate from blastema extracts contains a neurite outgrowth activity for PC12 cells and this activity is potentiated by heparin; the epidermal cap contains about twofold more activity than the mesenchyme of the corresponding blastemas.

Effect of Blastema Extracts on Proliferation of CCL39 Cells

This assay was performed with the 1.15 and 2.0 M eluates obtained from the 950 whole blastema extract (these fractions were also used for the following immunologic assays). The radioactivity in lysates from cultured CCL39 cells treated with 10 μ l of the 2 M eluate (with or without heparin) did not increase, indicating no mitogenic response. On the other hand, 10 or 20 μ l of the 1.15 M eluate enhanced the radioactivity of the lysate 6-fold with 20 μ l when used with heparin, and 1.5-fold without heparin (Fig. 1).

These results indicate that the neurite outgrowth activity detected in the 1.15 M eluate from heparin-Se-

pharose chromatography of blastema extracts is also mitogenic for fibroblasts and that this mitogenic activity is potentiated by heparin. Therefore this activity is generated by a factor which is different from the neurotrophic factor activity we extracted from blastemas, especially from mesenchyme (Boilly, 1989).

Immunologic Characterization of the Blastema Extracts

The ELISA test showed that the antibodies to aFGF but not bFGF bound to the 1.15 M fraction and that the highest immunoreactivity was found in the first three milliliters of elution; the three other milliliters of elution also exhibited a immunoreactivity but at a lower level (data not shown). On the other hand, the 2 M fraction did not show any binding with either antibody. Western blotting performed with the antibody against aFGF exhibited an immunoreactive major band with a molecular mass of 16 kDa for the 1.15 M fraction, co-migrating at roughly the same position with a preparation of aFGF from bovine brain (Fig. 2). A minor immunoreactive band which might correspond to a truncated form of FGF is also observed at about 15 kDa. Two other bands (about 31 kDa and 35 kDa) seen on the autoradiogram probably correspond to dimers formed by the first two bands (a second band is seen for bovine aFGF).

Scatchard Analysis

The Scatchard plot (Fig. 3) reveals the presence of two classes of specific binding sites for aFGF on blastema membranes; the high affinity binding sites have a disso-

FIG. 2. Immunoblot analysis of blastema heparin-binding proteins using anti-bovine aFGF antibodies. Blastema heparin-binding proteins eluted with 1.15 M NaCl were precipitated and electrophoresed on SDS/PAGE under reducing conditions and the proteins were electrophoretically transferred to immobilon membrane. The blot was then probed with anti-bovine aFGF and revealed with ¹²⁵I-protein A. Lane a, bovine aFGF; lane b, blastema heparin binding proteins.

FIG. 3. Scatchard analysis of competitive binding assay between ^{125}I -aFGF and unlabeled aFGF on blastema membranes. Blastema membranes (30 μg) were incubated (40 min, 4°C) with 23 pM of ^{125}I -aFGF and various concentrations (0 to 23 nM) of unlabeled aFGF.

ciation constant (K_d) of 25 pM and a membrane capacity of 30 fmole/mg of protein and the low affinity binding sites have a K_d of 18 nM and a membrane capacity of 30 pmole/mg of membrane protein.

Binding Assays of FGF on Blastema Sections

In order to localize the aFGF binding sites in blastemas we performed autoradiographic studies on blastema sections which were previously incubated with bovine ^{125}I -aFGF. We observed that ^{125}I -aFGF bound with the same affinity to the epidermal cap and the mesenchyme of the blastema. This binding was displaced by cold aFGF; the displacement occurred (Fig. 4a) more readily on mesenchyme than on epidermal cap, $P < 0.05$ for a 500-fold molar excess of cold aFGF. A 2 M NaCl or heparin treatment of the sections after incubation with the labeled factor decreased the binding to ^{125}I -aFGF on the blastemal tissues. The effect was greater for the epidermal cap than the mesenchyme (Fig. 4b). Heparinase pretreatment of the sections also decreased the binding on blastemal tissues; epidermal cap appeared more sensitive to this treatment. Chondroitinase treatment had no effect on the binding of ^{125}I -aFGF on the blastema sections (Fig. 4c).

Similar results were obtained with ^{125}I -bFGF (data not shown).

DISCUSSION

Our results suggest that regenerating limb blastemas contain an aFGF-like growth factor. This factor is eluted from heparin-Sepharose with 1.15 M NaCl, stimulates DNA synthesis of CCL 39 fibroblasts, and induces

neurite outgrowth of PC12 cells. These latter two activities are potentiated by heparin. The factor co-migrates on SDS-PAGE with bovine aFGF (Western blots), further indicating that this material is very similar if not identical to aFGF. As heparin-Sepharose chromatography was performed on extracts from either epidermal cap or blastemal mesenchyme, this growth factor is present in both of these blastemal compartments. These results agree with previous work (Boilly and Albert, 1990) which showed that crude extracts from the epidermal cap as well from blastemal mesenchyme were mitogenic for cultured blastema cells, although the stimulatory effect observed under these conditions may not be due entirely to the aFGF-like substance. Although no precise quantitation of the FGF was done (because of the small amount of material available), the stimulation obtained with the 1.15 M eluate from epidermal cap extracts always appeared higher than those obtained from the mesenchyme ones, suggesting that the epidermal cap of a blastema contains more aFGF than the underlying mesenchyme. We also observed that crude extracts from the epidermal cap were more potent for proliferation of blastema cells than extracts from mesenchyme (Boilly and Albert, 1990).

This is the first time that a well-defined growth factor has been identified in regenerating limb blastemas (Boilly, 1989). FGF has already been detected in the developing limb of chick embryos by competition with ^{125}I -aFGF binding to the FGF receptors of 3T3 cells (Seed *et al.*, 1988) and may be secreted by the posterior region (containing ZPA) of the limb bud mesoderm (Aono and Ide, 1988). Moreover bFGF has been characterized by Western blot analysis of the extracts of stage-18 chicken embryo limb buds using an antibody raised against a synthetic peptide corresponding to the 12 amino acids at the N-terminus of bFGF (Munaim *et al.*, 1988); on the other hand, bFGF expression has been detected at the highest levels in the developing limbs and tail of the 13.5-day mouse embryo (Hebert *et al.*, 1990). The fact that blastemas contain an aFGF-like material is interesting because this growth factor, contrary to bFGF which is widely distributed, is found only in a few tissues (Gospodarowicz and Ferrara, 1988). aFGF seems to be involved in hepatocyte regeneration where it is considered to be a candidate for the early autocrine stimulus for hepatocyte DNA synthesis (Kan *et al.*, 1989). The presence of aFGF in blastemas might also explain the stimulatory effect of heparin on cultured blastema cells (Boilly, 1989).

This factor probably functions during limb regeneration because blastema membranes contain specific high affinity binding sites which are considered to mediate the biological activity of FGF (Moscatelli, 1987). Such binding sites were described by Olwin and Hauschka

FIG. 4. Histoautoradiogram analysis of ^{125}I -aFGF binding on longitudinal sections of mid-bud stage blastemas of axolotls. The labeling is measured on the epidermal cap and the mesenchymal compartment of blastemas with the Samba 200 microscopic image processor. It is expressed as pixels ($0.4 \times 0.4 \mu\text{m}$) $\pm$ SD of silver grains in a 4096 pixels square ($625 \mu\text{m}^2$). (a) Blastema sections were incubated with $0.5 \text{ ng}/30 \mu\text{l}$ of iodinated aFGF in absence (control) or in presence of 500- or 1000-fold excess of unlabeled aFGF. (b) Blastema sections were washed with 2 M NaCl or heparin after iodinated aFGF binding. (c) Blastema sections were pretreated with heparinase or chondroitinase before iodinated aFGF binding.

(1990) in the limb of chick embryos; interestingly, the level of receptors we found in limb blastemas of axolotls is similar to that in the limbs of Day 6 to Day 9 chick embryos. We also have detected specific low affinity binding sites as has been seen from other membrane preparations (Courty *et al.*, 1988; Ledoux *et al.*, 1989; Hondermarck *et al.*, 1990); the function of these binding sites, known as heparin-like compounds, is still unknown (Moscatelli, 1987).

The problem is attempting to understand the function of this growth factor in the regenerating limb blastema. It is well known that FGF has different activities (mitogenic, angiogenic, neurotrophic, mesoderm induction) according to the nature of the target cell (Gospodarowicz and Ferrara, 1988). aFGF is probably not the neurotrophic factor which acts on transected nerves regenerating from the stump into the blastema, because the factor involved is produced only by the mesenchyme (Boilly, 1989); as the epidermal cap has a higher level of aFGF than does the blastemal mesenchyme and it is not neurotrophic for the transected nerves (Boilly, 1989),

aFGF probably does not function as this neurotrophic factor. As cell proliferation is one of the major phenomena characterizing early stages of limb bud regeneration, it is likely that aFGF is concerned with this, especially as it has been shown that FGF enhances mitotic activity of blastemas (Mescher and Gospodarowicz, 1979; Gospodarowicz and Mescher, 1980; Carlone *et al.*, 1981; Mescher and Loh, 1981; Mescher, 1983). We have also shown earlier that cultured blastema cells were responsive to a- or bFGF (Albert *et al.*, 1987). aFGF, a well-known angiogenic molecule, might also function in this fashion during regeneration, especially as angiogenesis has already been shown to be an important feature in limb regeneration (Smith and Wolpert, 1975; Revardel and Chapron, 1975).

The two possible functions (mitogenic, angiogenic) that we propose for aFGF during limb regeneration involve mesenchymal cells as targets for this growth factor. The results reported here showing FGF binding to blastema sections support this idea. The specificity of the fixation of ^{125}I -aFGF to blastema sections has been

ascertained by competitive binding experiments. This binding is heparinase sensitive, which indicates that at least a part of FGF binds to heparin-like structures. This is reinforced by the fact that heparin or a 2 M NaCl wash decreases the binding. Such results are similar to those observed on the mouse embryonic eye basement membrane (Jeanny *et al.*, 1987) and on rabbit corneal epithelial wounds (Assouline *et al.*, 1989).

Also interesting are the differences in binding characteristics we observed between the epidermal cap and the blastemal mesenchyme. The blastemal mesenchyme appeared less sensitive than the epidermal cap to a pretreatment with heparinase or to a wash in the presence of 2 M NaCl or heparin. Contrary to heparin-like structures, the high affinity receptors for FGF are less sensitive to these treatments (Moscatelli, 1987); therefore our results suggest the presence of high affinity receptors for FGF in mesenchyme. This hypothesis is in accord with the fact that ^{125}I -aFGF is more easily displaced by cold aFGF in mesenchyme, which suggests a higher affinity of mesenchyme compared to epidermal cap for this growth factor. This agrees with observations on the responsiveness of cultured blastema cells to a- or bFGF (Albert *et al.*, 1987).

The presence of low affinity binding sites in the epidermal cap could be related to the presence of a high level of heparan sulfate in this tissue (data not shown). The epidermal cap might therefore store the FGF material bound to the extracellular matrix as it does in different tissues (Baird and Ling, 1987; Vlodavsky *et al.*, 1987). The blastema mesenchyme which also contains heparan sulfate (data not shown) might also play such a function. The release of the FGF material from its storage sites would be important for the proliferation of blastema cells; unfortunately nothing is known about the regulation of FGF release during limb regeneration. In view of these findings, some old observations on the effect of saturated solutions of NaCl on amputated limbs of frogs (Rose, 1944, 1945) or mice (Neufeld, 1980) are interesting to consider. This treatment, which is able to initiate hypomorphic regeneration in frogs and partial blastema formation in mice, might cause the release of FGF from its heparin-like binding sites and allow it to stimulate cell proliferation. Obviously such a process cannot be considered for natural regeneration but the displacement of extracellular-matrix-bound FGF by heparin-like molecules and/or heparin sulfate degrading enzymes (Bashkin *et al.*, 1989) might be involved in this phenomenon.

Our results suggest that in regenerating limb blastemas aFGF has an epidermal origin and a mesenchymal target. This hypothesis is in accord with data related to the production of FGF by normal keratinocytes (Halaban *et al.*, 1988) and agrees with the fact that the epider-

mal cap delays chondrogenesis in regenerating limbs (Globus *et al.*, 1980) and that FGF stimulates the synthesis of a molecule involved in the control of chondrogenesis, namely, hyaluronic acid (Toole, 1982; Toole *et al.*, 1984) in both regenerating (Mescher and Munaim, 1986) and developing limbs (Toole *et al.*, 1989). It also explains the stimulation of proliferation of blastema cells with FGF when the wound epidermis of amputated limbs was replaced by a full thickness skin flap (Chew and Cameron, 1983). Nevertheless we cannot exclude the possibility of an autocrine stimulation of the epidermis because keratinocytes are known to be responsive to a- and bFGF (Ristow and Messmer, 1988; Shipley *et al.*, 1989) and to an aFGF-related molecule (Rubin *et al.*, 1989; Finch *et al.*, 1989). An FGF autocrine stimulation of proliferation might also involve the mesenchymal cells. Such a mechanism has already been described for FGF in fast growing tissues like capillary endothelial cells (Moscatelli *et al.*, 1988; Schweigerer *et al.*, 1987) vascular smooth muscle cells (Winkles *et al.*, 1987), and regenerating hepatocytes (Kan *et al.*, 1989). We need now to study the expression of FGF and FGF receptor genes as well as the immunolocalization of their products at the cellular level to resolve this problem.

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