Roux's Arch Dev Biol (1990) 198:443–447

Roux's Archives of
Developmental
Biology

In vitro control of blastema cell proliferation by extracts from epidermal cap and mesenchyme of regenerating limbs of axolotls

Bénoni Boilly and Philippe Albert

Laboratoire de Biologie des Facteurs de Croissance, Université de Lille, France

Received October 23, 1989 / Accepted in revised form February 20, 1990

Summary. The presence of a mitogenic activity in limb blastemas of axolotls was detected in crude extracts of blastemas at the mid-bud stage. The mitogenicity of the extracts was estimated from the mitotic index of blastema cells grown for 6 days in the presence of limb blastema extracts, with colchicine present for the last 2 days. All the extracts tested (whole blastema, blastemal mesenchyme, epidermal cap) significantly enhanced proliferation of blastema cells. The highest stimulation factors we observed were 7 × with 7 μg protein/ml whole blastema extracts, 5.2 × with 14 μg/ml blastemal mesenchyme extracts, and 11 × with 3.5 μg/ml epidermal cap extracts. Hence the epidermal cap extracts appeared to be the most mitogenic. Extracts from the blastemal mesenchyme, although less mitogenic than the epidermal cap extracts, were more potent than nerve extracts [Albert P, Boilly B (1986) Biol Cell 58:251–262]. These results are discussed with regard to the production of growth factors during limb regeneration.

Key words: Limb regeneration – Proliferation – Blastema – Epidermal cap – Axolotl – Growth factors

Introduction

After amputation, limbs of urodeles regenerate completely. Regeneration begins with wound healing, accumulation of mesenchymal cells beneath the wound epidermis, and invasion of the mesenchymal cell mass by regenerating nerves (see Wallace 1981 for review). Proliferation leads to the formation of a blastema, which is composed of an epidermis (epidermal cap) surrounding the mass of mesenchymal blastema cells (mesenchyme). Regeneration of a limb depends on the proliferation of the blastema cells, so any treatment that blocks proliferation, such as X-irradiation, denervation or removal of the epidermal cap (see Wallace 1981 for review), will stop the regeneration process. It is important to understand what controls the proliferation of blastema cells in order to comprehend better the process of regeneration. Excluding systemic factors like hormones (see for review Liversage et al. 1985), the three components of blastemas (i.e. regenerating nerves, epidermal cap, mesenchyme) can be considered as potential sources of mitogens. The production of growth factor(s) by nerve tissue during regeneration has been demonstrated by Singer (1974, 1978) and others (see Carlone and Mescher, 1985 for review) but its nature is still unknown.

Less is known about the epidermal cap and mesenchyme. Mescher (1976) suggested, 'The epidermal cap may serve to keep the dedifferentiating cells in the cell cycle and prevent their early redifferentiation'. Using culture of explanted blastemas, Globus et al. (1980) were able to demonstrate that the epidermal cap controlled the cycle of mesenchymal blastema cells; after removal of the epidermal cap, mesenchymal cells left the cell cycle and differentiated precociously into cartilage nodules. Globus et al. (1980) suggested that the epidermal cap provided a 'division signal' for mesenchymal cells, which differentiate in its absence. The production of this 'signal' would allow for a high proliferation rate in blastema cells just beneath the epidermal cap. Cells further from the epidermal cap would be exposed to decreasing concentrations of the signal, and would therefore differentiate. However, as pointed out by Globus and Vethamany-Globus (1985), 'The manner in which the AEC (apical epidermal cap) exerts its effect has not been determined'. There are no experiments relating to the possible mitogenic influence of blastemal mesenchyme on itself during regeneration. Although several authors have used limb blastema extracts on denervated (Deck and Futch 1969; Burnett et al. 1971) or X-irradiated limbs (Deck and Dent 1970) their results are difficult to interpret because they used extracts prepared from whole blastemas, and because these extracts were tested in vivo. Testing crude extracts in vivo induces inflammatory re-

Offprint requests to: B. Boilly, Laboratoire de Biologie des Facteurs de Croissance, Université de Lille I, F-59655 Villeneuve d'Ascq Cedex, France

sponses, which can interfere with the effect of any potent growth factor contained in the extract. To search for mitogens in tissues, we need a sensitive bioassay using cultured blastema cells. We have developed such a bioassay (Albert and Boilly 1986) and have used it to confirm the presence of mitogens in nerve tissue of amphibians; we have also shown that the production of mitogens by the spinal cord is regulated during regeneration (Boilly and Albert 1988). In this paper, we use the same bioassay to test the mitogenicity of extracts from the epidermal cap and from the mesenchyme. We show that extracts from the epidermal cap of limb blastema are highly mitogenic and that extracts from the mesenchyme, although clearly less potent, are more mitogenic than the previously studied nerve extracts.

Materials and methods

Mid-bud stage blastemas were obtained from 9–12-month-old axolotls (*Ambystoma mexicanum*), the forelimbs of which were amputated under anesthesia (MS 222 0.1% Sandoz) through the distal stylopod. After peeling off the epidermal cap, the mesenchyme was mechanically dissociated with needles in culture medium and the mesenchymal cells plated in petri dishes (35 mm diameter, Falcon Primaria). The culture medium was made with diluted MEM (Seromed) serum-free, complemented with insulin, L-thyroxine, somatotropin, hydrocortisone and antibiotics (osmolarity 250/260 mOsm, pH 7.3) as described previously (Albert and Boilly 1986). The cultures were maintained for 16 days at 25° C (2% CO_2, 98% air) and the assays of the mitotic index were conducted only on the layer of blastema cells that had migrated from the explants (Albert and Boilly 1986).

The mitogenic activity of extracts was tested as follows. The extracts were added to the culture medium of seven cultures on the 10th day of culture; on the 14th day, the medium was removed and extracts added again at the same concentration with 20 µg/ml colchicine (Serva, Heidelberg). The mitotic index was established 48 h later. About 500–1000 cells were examined in each dish in order to calculate the mitotic index. All the experiments were performed in replicate.

Extracts were obtained from mid-bud stage blastemas of axolotls 14 days after forelimb amputation or from muscles (*musculus dorsalis tronci*). The blastema extracts were prepared either from the epidermal cap, the mesenchyme, or the whole blastema. We checked histologically that the epidermal cap and the mesenchyme we isolated from blastemas were pure (Fig. 1). All the samples were maintained in Ringer's solution at 0° C. They were homogenized (Potter homogenizer) and centrifuged (25000 *g*, 1 h, 4° C) and the supernatant was removed. The protein content of the supernatant was determined (Bradford 1976) prior to bioassay; the quantity of protein in the supernatant used varied between 3.5 µg/ml and 28 µg/ml culture medium. As a control, bovine serum albumin was added to cultures in similar amounts.

Results and discussion

Our results show that crude extracts from blastemas, particularly from the epidermal cap, strongly stimulate the proliferation of cultured mesenchymal blastema cells. The absence of an influence of bovine serum albumin on the one hand, and the low level of stimulation obtained with a non-blastemal tissue (muscle) on the other hand (Fig. 2), show that the stimulatory effect of tissue extracts is not a consequence of the addition of proteins to the culture medium (when used at a concentration similar to that of blastema extracts: 3.5–14 µg protein/ml); rather the effect we obtained is correlated with the blastemal origin of the extracts. The blastema extracts used appeared to be mitogenic; they stimulated the proliferation of blastema cells up to a maximum concentration that depended on the tissue origin of the extract; beyond this concentration, the stimulation of cell proliferation decreased.

Extracts of epidermal cap were highly mitogenic. The mitotic index reached its maximum level (19.6% vs 1.8% for the control) at a relatively low concentration of protein (3.5 µg/ml) (Fig. 3). There is already some indirect evidence that the epidermal cap secretes a product into the underlying blastema. Singer and Salpeter (1961) presented electron micrographs of epidermal cells, the appearance of which suggested secretion; Chapron (1974) using [^{3}H]fucose labeling showed that the epidermal cap synthesizes a glycoprotein that localizes between blastema cells; Revardel et al. (1986) reported that extracts from scar epithelium increase digit regeneration of *Rana*. However, no evidence concerning the mitogenicity of an epidermal secretion has previously been presented. Our results show that the mitogenic activity appears to be very effective since a quantity of epidermal cap extract that is ten times less (measured by its protein content) than that used for nerve extracts (Albert and Boilly 1986) is twice as mitogenic. The nature of this mitogenic activity is at present unknown. However, Chew and Ca-

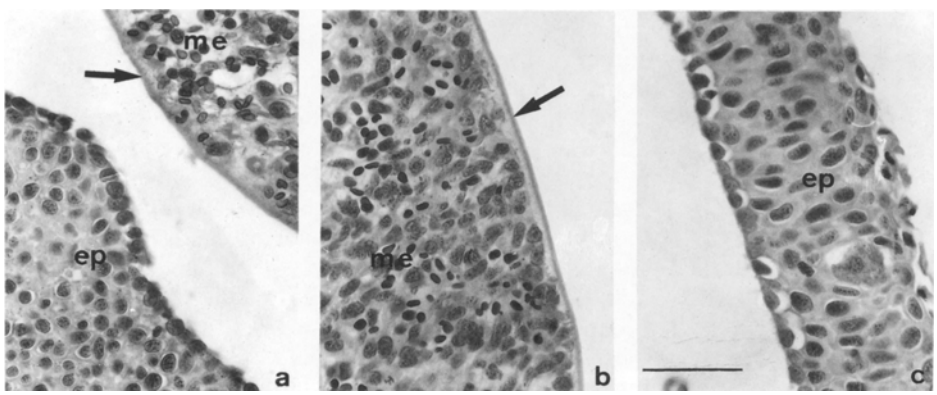

Fig. 1. Histological section of a blastema, the mesenchyme (*me*) and the epidermal cap (*ec*) of which are partially separated (**a**) or totally separated (**b**, **c**); note that the basement membrane (*arrow*) is present on the mesenchyme after separation. Bar: 100 µm

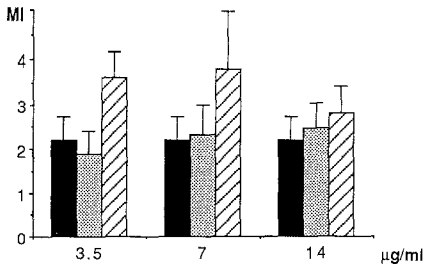

Fig. 2. Influence of bovine serum albumin or muscle extract (µg/ml culture medium) on the mitotic index (*MI*) of blastema cells. The mitotic index represents the number of mitoses for 100 cells. ■ Control; ▨ Bovine serum albumin; ▨ Muscle

meron (1983) succeeded in stimulating the proliferation of dedifferentiating stump cells in amputated limbs deprived of the wound epidermis by implanting Elvax 40 impregnated with crude preparations of fibroblast growth factor (FGF) or endothelial cell growth supplement (ECGS). The mitotic indices in the area of dedifferentiation of animals receiving implants increased by a factor of six for ECGS and ten for FGF over controls without implants. Although we cannot know from these experiments if the growth factors replace whatever is normally supplied by the epidermal cap, it has been shown in separate studies that the proliferation of blastema cells can be stimulated by FGF (Mescher and Gospodarowicz 1979; Rathbone et al. 1979; Gospodarowicz and Mescher 1980; Carlone et al. 1981; Mescher and Loh 1981; Albert et al. 1987). In addition, we have recently succeeded in extracting acidic FGF from the epidermal cap of axolotls (Boilly 1989).

Extracts of mesenchyme alone also stimulated proliferation of mesenchymal blastema cells. However, the maximum efficiency (5.2× the controls) was less than half that of epidermal cap extracts and moreover required the fourfold concentration (14 µg/ml as compared to 3.5 µg/ml) (Fig. 3). Since blastema cells can be stimulated to divide by both nerves and epidermal cap, it is possible that the mitogenicity of blastema cell extracts is provided by the mitogens from the nerve and/or epidermal cap, which can be sequestered outside the cell, in the extracellular matrix like FGF (Jeanny et al. 1987; Vlodasky et al. 1987a, b; Baird and Ling 1987). It is also possible that the mitogen we obtained in extracts of mesenchymal blastema cells is produced by the blastema cells themselves. Such an autocrine control of growth is known for FGF in fast-growing cells like cancer cells (Sporn and Roberts 1985) or in normal cells able to undergo rapid histogenesis, such as human vascular smooth muscle cells (Winkles et al. 1987) and capillary endothelial cells (Moscatelli et al. 1986; Schweigerer et al. 1987). Moreover, recent results related to the stimulation of blastema cells by heparin (unpublished data) suggest that blastema cells produce an acidic FGF-like growth factor, a factor we have also extracted from mesenchyme during regeneration (Boilly 1989). In addition, other growth factors such as basic FGF could be produced by specialized cells like macrophages (Baird et al. 1985, 1986), which are known to be present in blastemas (Bryant et al. 1971).

Extracts obtained from the whole blastema enhanced proliferation of mesenchymal blastema cells at a high level; maximum stimulation (5.2× to 7×) was obtained for a concentration of 7 µg protein/ml (Fig. 3). It appears that the mitogenic effect we observed with extracts from whole blastemas results from the activity of both components of the blastema without potentiation or inhibition, since extracts from whole blastemas are more potent than mesenchyme extracts and less potent than epidermal cap extracts, and since the epidermal cap represents about half of the whole blastema (in cell number).

The mitogenic effect of limb blastema extracts has already been tested in vivo on denervated (Deck and Futch 1969; Burnett et al. 1971) or X-irradiated (Deck and Dent 1970) limbs. These extracts, which were considered by Burnett et al. (1971) to contain the mitogen furnished by nerves, allowed denervated limbs to produce an early blastema in 76% of the cases (Deck and Futch 1969) or to 'maintain normal regeneration' (Burnett et al. 1971) but did not stimulate recovery of irradiated limbs (Deck and Dent 1970). In a similar manner, Skowron et al. (1963), Semkowicz (1964), Weber and Maron (1965), and Wiecek (1966) observed that tail blastema homogenates accelerated the growth of regenerating tails. However, the effect of infusion of crude extracts

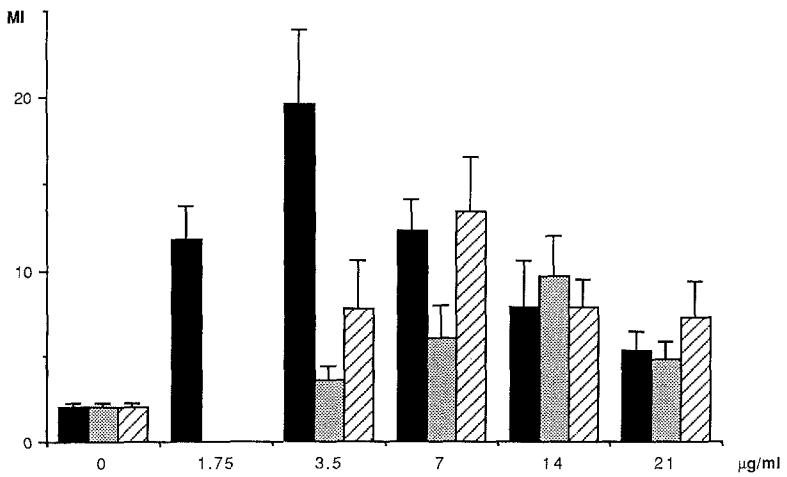

Fig. 3. Influence of whole blastema extract, mesenchyme extract, or epidermal cap extract (µg/ml culture medium) on the mitotic index (*MI*) of blastema cells. The mitotic index represents the number of mitoses for 100 cells. ■ Epidermal cap; ▨ Mesenchyme; ▨ Blastema

in vivo is rather difficult to interpret since the administration of various tissue or tissue extracts can stimulate regeneration (Malinin and Deck 1958; Polezhaev 1959; Polezhaev and Ermakova 1960; Polezhaev et al. 1961, 1964; Polezhaev and Tuchkova 1967; Smith and Crawford 1969); it is probable that this stimulation of regeneration resulted more from an inflammatory response (which is known to generate growth factors; Turck et al. 1987) than from the 'trophic' factor, which might be contained in the extract, because infusion of saline (Ringer's solution) also produced a positive effect on growth (Deck 1971). On the other hand, because of the type of bioassay used in this work (cell culture), our results allow us to assume that blastemas contain mitogens. In the same way, Gospodarowicz and Mescher (1981) reported that crude extracts from regenerating limbs of axolotls exhibited an FGF-like mitogenic effect on mouse 3T3 cells, and Brockes (1984) and Brockes and Kintner (1986) using another target cell (rat Schwann cell) showed that blastemas of *Notophthalmus viridescens* contained glial growth factor, a growth factor related to basic FGF (Gospodarowicz et al. 1987). Although some of the growth factors present in blastemas are furnished by nerves, as proven by Brockes (1984) and Brockes and Kintner (1986) for glial growth factor, our results suggest that they can also originate from other sources, like the epidermal cap and perhaps from the blastema cells themselves. This explains why denervation of a regenerating limb does not completely stop the proliferation of blastema cells (Boilly et al. 1985) and why nerve extracts are less potent than blastema extracts.

References

Albert P, Boilly B (1986) Evolution in vitro de cellules de blastèmes de régénération de membre d'Axolotl: influence de l'insuline et d'extraits nerveux sur la prolifération cellulaire. Biol Cell 58:251–262

Albert P, Boilly B, Courty J, Barritault D (1987) Stimulation in cell culture of mesenchymal cells of newt limb blastemas by EDGFI or II (basic or acidic FGF). Cell Differ 21:63–68

Baird A, Ling N (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase like enzymes in the neovascular response. Biochem Biophys Res Commun 142:428–435

Baird A, Mormede P, Böhlen P (1985) Immunoreactive fibroblast growth factor in cells of peritoneal exsudate suggests identity with macrophages derived growth factor. Biochem Biophys Res Commun 126:358–364

Baird A, Esch F, Mormede P, Ueno N, Ling N, Böhlen P, Ying SY, Wehrenberg WB, Guillemin R (1986) Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. Rec Prog Horm Res 42:177–205

Boilly B (1989) Production of growth factors by the blastema during limb regeneration of urodeles (Amphibia). In: Kiortsis V, Koussoulakos S, Wallace H (eds) Recent trends in regeneration research. Plenum Press New York, pp 91–96

Boilly B, Albert P (1988) Blastema cell proliferation in vitro: effects of limb amputation on the mitotic activity of spinal cord extracts. Biol Cell 62:183–188

Boilly B, Oudkhir M, Lassalle B (1985) Control of the blastemal cell cycle by the periferal nervous system during newt limb regeneration: continuous labeling analysis. Biol Cell 55:10' 112

Bradford MM (1976) A rapid and sensitive method for the quant tation of microgram quantities of protein utilizing the princip of protein-dye binding. Anal Biochem 72:248–254

Brockes JP (1984) Mitogenic growth factors and nerve dependen of limb regeneration. Science 225:1280–1287

Brockes JP, Kintner CB (1986) Glial growth and nerve-depende proliferation in the regeneration in the blastema of urodele an phibians. Cell 45:301–306

Bryant SV, Fyfe D, Singer M (1971) The effects of denervatic on the ultrastructure of young limb regenerates in the new *Triturus*. Dev Biol 24:577–595

Burnett AL, Kary CE, Lagorio AM (1971) Induction of growt in newt and frog limbs after perfusion with extracts from nev blastemas. Nature 234:98–99

Carlone RL, Mescher A (1985) Trophic factors from nerves. I Sicard R-E (ed) Regulation of vertebrate limb regeneratio Oxford University Press, Oxford, pp 93–105

Carlone RL, Ganagarajah M, Rathbone MP (1981) Bovine pitu tary fibroblast growth factor has neurotrophic activity for new limb regenerates and skeletal muscle in vitro. Exp Cell Re 132:15–21

Chapron C (1974) Mise en évidence du rôle, dans la régénératio des Amphibiens, d'une glycoprotéine secrétée par la cape ap cale: étude cytochimique et autoradiographique en microscop électronique. J Embryol Exp Morphol 32:133–145

Chew KE, Cameron JA (1983) Increase in mitotic activity of reger erating axolotl limb by growth factor-impregnated implant J Exp Zool 222:325–329

Deck JD (1971) The effects of infused materials upon the regener tion of newt limbs. II. Extracts from newt brain and spin cord. Acta Anat 79:321–332

Deck JD, Dent JN (1970) The effects of infused materials upo the regeneration of newt limbs: III. Blastemal extracts and alk line phosphatase in irradiated limb stumps. Anat Rec 168:525 536

Deck JD, Futch CB (1969) The effects of infused materials upo the regeneration of newt limbs: I. Blastemal extracts in dene vated limb stumps. Dev Biol 20:332–348

Globus M, Vethamany-Globus S (1985) In vitro studies of contro ling factors in Newt limb regeneration. In: Sicard R-E (e Regulation of vertebrate limb regeneration. Oxford Universit Press, Oxford, pp 106–127

Globus M, Vethamany-Globus S, Lee YCI (1980) Effect of apic epidermal cap on mitotic cycle and cartilage differentiation i regeneration blastemata in the newt *Notophthalmus viridescen* Dev Biol 75:358–372

Gospodarowicz D, Mescher AL (1980) Fibroblast growth facto and the control of vertebrate regeneration and repair. An Nev York Acad Sci 339:151–172

Gospodarowicz D, Mescher AL (1981) Fibroblast growth facto and vertebrate regeneration. Adv Neurol 29:149–171

Gospodarowicz D, Neufeld G, Schweigerer L (1987) Fibrobla growth factor: structural and biological properties. J Cell Phy iol [Suppl] 5:15–26

Jeanny JC, Fayein N, Moenner M, Chevallier B, Barritault D Courtois Y (1987) Specific fixation of bovine brain and retin acidic and basic fibroblast growth factors to mouse embryoni eye basement membranes. Exp Cell Res 171:63–75

Liversage RA, Mc Laughlin DS, Mc Laughlin HMG (1985) Th hormonal milieu in amphibian appendage regeneration. In: S card R-E (ed) Regulation of vertebrate limb regeneration. O ford University Press, Oxford, pp 54–80

Malinin T, Deck JD (1958) The effects of implantation of embryo ic and tadpole tissues into adult frog limbs. I. Regeneratio after amputation. J Exp Zool 139:307–328

Mescher AL (1976) Effects on adult newt limb regeneration partial and complete skin flaps over the amputation surfac J Exp Zool 195:117–127

Mescher AL, 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 AL, Loh JJ (1981) Newt forelimb regeneration blastema in vitro: cellular response to explantation and effects of various growth-promoting substances. J Exp Zool 216:235–245

Moscatelli D, Presta M, Joseph-Silverstein J, Rifkin DB (1986) Both normal and tumor cells produce basic fibroblast growth factor. J Cell Physiol 129:273–276

Polezhaev LV (1959) Restoration of regenerative ability of extremities of axolotls after irradiation with roentgen rays. Dokl Akad Nauk SSSR 127:713–716

Polezhaev LV, Ermakova NI (1960) Restoration of regenerative capacity of the extremities in axolotls depressed by roentgen radiation. Dokl Akad Nauk SSSR 131:209–212

Polezhaev LV, Tuchkova S, Ya (1967) Restoration of regenerative power of axolotl limbs inhibited by X-ray irradiation with tissue heterografting. Dokl Akad Nauk SSSR 180:754–757

Polezhaev LV, Teplits NA, Ermakova NI (1961) Restoration of the regenerative property of extremities in axolotls inhibited by X-ray irradiation, by means of proteins, nucleic acids and lyophilized tissues. Dokl Akad Nauk SSSR 138:477–480

Polezhaev LV, Teplits NA, Tuchkova S, YA (1964) Restoration of regenerational capacity of extremities of axolotls after it has been suppressed by X-rays with the aid of nucleid acids. Dokl Akad Nauk SSSR 159:682–685

Rathbone MP, Carlone RL, Ganagarajah M (1979) Neurotrophic like effects of bovine pituitary fibroblast growth factor on regenerating limb blastema and skeletal muscles of newts in vitro. Am Zool 19:925

Revardel JL, Aoussi S, Chapron C (1986) Actions d'extraits d'épithélium cicatriciel sur la régénération des doigts chez *Rana ridibunda* adulte: étude histologique et biométrique. Can J Zool 64:2683–268

Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325:257–259

Semkowicz J (1964) Effect of X-rays on the inhibition of promoting action of the homogenate of regeneration blastemas. Folia Biol (Krakow) 12:383–414

Singer M (1974) Neurotrophic control of limb regeneration in the newt. Ann NY Acad Sci 228:308–322

Singer M (1978) On the nature of neurotrophic phenomenon in urodele limb regeneration. Am Zool 18:829–841

Singer M, Salpeter M (1961) Regeneration in vertebrates: the role of the wound epithelium. In: Zarrow M-X (ed) Growth in living systems. Basic Books, New York, pp 277–311

Skowron S, Rzehak K, Maron K (1963) The effect of blastemal tissue on regeneration. Folia Biol (Krakow) 7:99–112

Smith St D, Crawford GL (1969) Initiation of regeneration in adult *Rana pipiens* limbs by injection of homologous liver nuclear R.N.P. Oncogenesis 23:299–307

Sporn MB, Roberts AB (1985) Autocrine growth factors and cancer. Nature 313:745–747

Turck CW, Dohlman JG, Goetzl EJ (1987) Immunological mediators of wound healing and fibrosis. J Cell Physiol [Suppl] 5:89–93

Vlodasky I, Folkman J, Klagsbrun M (1987a) Heparin-binding endothelial cell growth factors are sequestered by the extracellular matrix. J Cell Biochem [Suppl] 35:58–64

Vlodasky I, Folkman J, Sullivan R, Friedman R, Ishai-Michaeli R, Sasse J, Klagsbrun M (1987b) Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 84:2292–2296

Wallace H (1982) Vertebrate limb regeneration. Wiley, Chichester. 276 p

Weber W, Maron K (1965) The effect of blastemal tissue on regeneration. II. Influence of blastema cell fractions on regenerative growth. Folia Biol (Krakow) 13:383–396

Wiecek M (1966) Further observations on the stimulating effect of blastemal tissue on regeneration. Folia Biol (Krakow) 14:229

Winkles JA, Friesel R, Burgess WH, Howk R, Mehlman M, Weinstein R, Maciag T (1987) Human vascular smooth muscle cells both express and respond to heparin-binding growth factor I (endothelial cell growth factor). Proc Natl Acad Sci USA 84:7124–7128