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Original Article

Developmental regulation of acidic fibroblast growth factor (aFGF) expression in bovine retina

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ABSTRACT Acidic fibroblast growth factor (aFGF) is a signalling molecule implicated in a wide variety of biological processes such as cell growth, differentiation and survival. It has been purified from bovine retina. The present study was carried out to detect which cells in the bovine retina expressed aFGF at the different stages of embryonic and post-natal development. The specific aFGF mRNA and protein were detected by *in situ* hybridization employing riboprobes and immunocytochemistry using affinity purified polyclonal human recombinant aFGF antibodies respectively. No signal was detected by either technique until 4-5 months and then there was progressive expression of aFGF with terminal morphogenesis of the retina. By 8-9 months of embryonic development, nuclei of the 3 neuronal layers (ganglion cell layer, inner and outer nuclear layers) were all uniformly and intensely labeled. A slight labeling of the pigmented epithelium of the retina was also visible throughout development and maturation. These results showed a good correlation between message and protein expression in these cell types. In contrast, glial cells in the nerve fiber layer and vascular endothelial cells displayed a nuclear immunostaining for the protein in the absence of message. These data suggest that aFGF plays a role in the late steps of retinal differentiation by autocrine and paracrine mechanisms.

KEY WORDS: *acidic fibroblast growth factor, in situ hybridization, immunohistochemistry, bovine, retina*

Introduction

Fibroblast growth factors (FGFs) constitute a family of mitogenic polypeptides having a high affinity for heparin. The family consists of acidic and basic FGFs (a and b FGF respectively) and five other members: FGF3 (int 2), FGF4 (hst-kFGF), FGF5, FGF6, and FGF7 (KGF). All these proteins share 30 to 50% homology in their aminoacid sequence (review in Burgess and Maciag, 1989). aFGF is a single chain polypeptide with a molecular weight of 15 to 18 kDa and a pI of 5.6 to 6.0. The aminoacid sequence has been completely determined (Burgess *et al.*, 1985). The unique aFGF coding gene generates mRNAs of different sizes (9.9, 6.0, 4.2, 1.0 kb) (Alterio *et al.*, 1988). aFGF is the predominant form in the central nervous system and in ocular tissues, especially the retina (Arruti and Courtois, 1978; Barritault *et al.*, 1981).

The retina is a complex nervous structure derived from a lateral evagination of the diencephalon consisting of several neuronal layers: the photoreceptors (rods and cones), the inner nuclear layer (cell bodies of bipolar, horizontal and amacrine cells) and the ganglion layer (cell bodies of ganglion cells). Besides the neuronal cell layers, there are numerous neuroglial and vessel associated cells. There are three main classes of glial cells: astrocytes, microglia (both restricted in most species to the nerve fiber layer and the inner and outer plexiform layers) (Schnitzer, 1988), and Müller cells. The latter are easily distinguishable by their nuclei located in either the inner or the outer nuclear layers (Blanks, 1982). The cellular organization and morphology of the adult retina results from a complex series of interacting developmental events including proliferation, migration, differentiation and survival. These events represent a specific spatio-temporal pattern (Blanks, 1982; Barnstable *et al.*, 1988).

Growth factors including several members of the FGF family have been implicated in some of these processes (review in Hicks

Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; EDGF, eye derived growth factor; FGF, 1,2,3,4,5,6, fibroblast growth factors 1,2,3,4,5,6; FGFRs, fibroblast growth factor receptors; GFAP, glial fibrillary acidic protein; KGF, keratinocyte growth factor; OCT, optimum cutting temperature; PBS, phosphate buffered saline; RPE, retina pigmented epithelium; SSC, saline sodium citrate.

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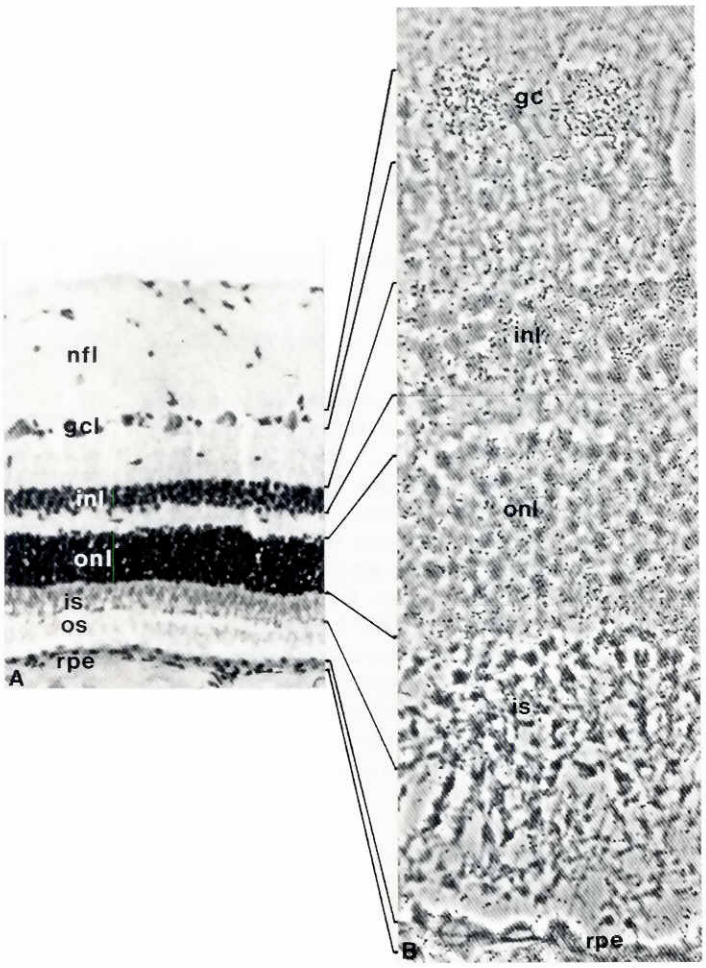

Fig. 1. aFGF *in situ* hybridization on adult retina. *Retina sections from 7-year-old bovine stained with hematoxylin-eosin* **(A)** *or hybridized with specific aFGF antisense riboprobe* **(B)**. **(A)** *Bright field (x180);* **(B)** *phase contrast (x600). The labeling is mainly localized on the ganglion cells (g c) and on the inner (inl) and outer nuclear layers (onl). A few grains are also visible on the inner segments of the photoreceptors (is) and on the retinal pigmented epithelium (r p e). gcl, ganglion cell layer; nfl, nerve fiber layer; os, outer segments of the photoreceptors.*

et al., 1991). Int 2 is expressed transiently in the rat during midgestation (Wilkinson *et al.*, 1989) while aFGF and bFGF have been extracted from 11 and 18 day old chick embryos (Mascarelli *et al.*, 1987). Recently, aFGF (Jacquemin *et al.*, 1990) and bFGF mRNAs (Noji *et al.*, 1990) have been described in adult bovine and rodent retina. Reports have also shown the presence of aFGF in the rod outer segment of the photoreceptors (Plouët *et al.*, 1988; Mascarelli *et al.*, 1989) and its ocular distribution (Caruelle *et al.*, 1989; De longh and McAvoy, 1992). The retina also contains specific high and low affinity FGF receptors (FGFRs) localized to the synaptic regions (inner and outer plexiform layers, outer limiting membrane) (Jeanny *et al.*, 1987; Cirillo *et al.*, 1990; Fayein *et al.*, 1990). In addition many *in vitro* and *in vivo* studies have reported the effects of FGFs on retinal biology and physiology (Sievers *et al.*, 1987; Hicks and Courtois, 1988, 1992; Lipton *et al.*, 1988; Plouët *et al.*, 1988; Guillemot and Cepko, 1992). Park and Hollenberg (1991) have shown exclusively in early chick embryos that aFGF can induce the neural retina regeneration from the overlying pigmented epithelium.

Despite the presence of aFGF and FGFRs and the effects of aFGF in highly specialized retinal cell types, the spatio-temporal expression of aFGF during embryonic and post-natal development is not known. Since the amount of aFGF mRNA in rodent is very low, making *in situ* hybridization difficult, in the present study, using *in situ* hybridization and immunohistochemical procedures, we studied aFGF expression in the developing and mature bovine retina.

Results

Localization of aFGF expression in the embryonic and adult retina

In situ hybridization of 7-year-old bovine retina sections using homologous aFGF riboprobes demonstrates a differential labeling of the retinal cells (Fig.1). The large ganglion cells are the most densely labeled, followed by cells of the inner and outer nuclear layers which contain all the photoreceptor nuclei. The glial and vascular endothelial cells, above the ganglion cell layer, identified by GFAP and Factor VIII antibodies respectively (data not shown), do not appear labeled. There is a slight but significant labeling of the RPE cells, which is apparent despite the presence of pigment within the cells. In all the tissues examined the sense probe gave no significant signal. Thus, most neuronal cells actively express aFGF mRNA.

The developmental regulation of aFGF expression was analyzed in 86 day, 4-5 month, 8-9-month-old calf embryos and 5-month, 3- and 7-year-old bovine retina. To precisely match the timing of appearance of aFGF expression with the stage of development (and also as an internal standard), consecutive sections were hybridized with aFGF and opsin probes. It has been shown previously that opsin, specifically expressed in the photoreceptors, appears late in ocular development (Brann and Young, 1986). As shown in Fig. 2A, in 86 day old calf embryo there was no specific labeling of the neuroepithelial layers for either probe. The grains observed on the retinal pigmented epithelial layers are not silver grains but pigments which are easily distinguishable by microscopic examination and are present at all stages of development. At 4-5 months of embryonic development a similar pattern is observed (Fig. 3A). a FGF expression becomes detectable at 8-9 months (Fig. 3B) just before birth. Three further time points were assessed, 5 months postnatal (Fig. 2B) representing the completion of retinal development, and 3 and 7 years (Figs. 2C, 3C). At these stages aFGF message is expressed mostly in the ganglion cells and considerably more than in the other cell layers, as described in Fig. 1. It is noteworthy that in addition to the neuronal layers, some expression can be observed in the vessel wall localized at the most inner part of the retina under the inner limiting membrane. As expected, opsin expression is localized only to the inner nuclear layer and the inner segments of the photoreceptors of mature retina (Fig. 2).

aFGF immunolocalization in the retina

The localization of aFGF was performed using an immuneserum prepared against recombinant human aFGF, purified by affinity on an aFGF immobilized column after chromatography on a human recombinant bFGF column to eliminate all cross reactions with bFGF. This purified antibody has been characterized and used successfully in other studies involving the optic nerve (Faucheux *et al.*, 1991a). In the adult retina, this antibody labels specifically the neuronal cell layers as well as the glial cells and the vascular

endothelium present at the level of the nerve fiber layer. The immunoreactivity is higher in ganglion cells and in the inner nuclear layer than in the photoreceptors. Pre-immuneserum as well as aFGF preabsorbed antibodies do not stain these layers. A strong autofluorescence is present in the RPE and in the rod segments (Fig. 4). In the developing retina of the 86 day old calf embryo (Fig. 5A), there is a slight labeling of the neuroepithelium, more pronounced on nascent ganglion cells. At the later stages (5-month-old calf and 3-year-old cow), the immunolabeling clearly detects aFGF in the cell body and the nuclei of all neuronal layers as well as in glial and capillary cell nuclei and in the vessel walls located through the nerve fiber layer (Fig. 5B,C). However, there was no labeling of the inner limiting membrane and Bruch's membrane. The inner nuclear layer as well as ganglion cells are more intensely labeled than the outer photoreceptor layer. Controls using the non-retained fraction from the affinity column are negative on these cells. In order to check if this localization is not due to a special antibody batch, we used another batch which gave similar results (data not shown). All neuronal cells, and nuclei of the glial and vascular endothelial cells located just underneath the inner limiting membrane were also positive.

Discussion

In these experiments, we used *in situ* hybridization and immunocytochemistry to follow the expression of aFGF in the developing bovine retina. As previously described (Alterio *et al.*, 1988; Jacquemin *et al.*, 1990), our data confirm that aFGF mRNA, as well as the aFGF protein formerly described by our laboratory as EDGF (eye derived growth factor) (Arruti and Courtois, 1978) and later identified as aFGF and bFGF (Baird *et al.*, 1985; Courty *et al.*, 1985; Schreiber *et al.*, 1985), is relatively abundant in the adult bovine retina. In the adult, the localization of the protein is mostly in or around the nucleus, a result confirming some of our preliminary work (Hicks *et al.*, 1991) but in contradiction with others. A membranous localization of aFGF was reported in the adult retinal rat ganglion cells (Elde *et al.*, 1991) while in the developing rat embryo, immunoreactivity with aFGF antibody was observed in all cell layers of the retina (Fu *et al.*, 1991). By differential centrifugation of bovine retinal membranes, we found that aFGF was mainly present in the rod outer segment (Plouët *et al.*, 1988). This is not apparent in the study presented here. aFGF has also been localized in the nuclei of optic nerve (Faucheux *et al.*, 1991a), spinal cord (Tourbah *et al.*, 1991, 1992), as well as a mixed (nuclei and cytoplasmic) localization in the subcortical neurons (Stock *et al.*, 1992). This raises the question of the subcellular localization of aFGF as well as its different forms. Despite the fact that aFGF has a nuclear targeting sequence (Imamura *et al.*, 1988), recent data indicate that aFGF may contain an additional sequence which prevents endogenously expressed aFGF from being translocated into the nucleus (Zhan *et al.*, 1992). Thus, the precise subcellular localization of aFGF, like that of bFGF, is still an open question (Kardami *et al.*, 1990; Renko *et al.*, 1990; Hanneken and Baird, 1992). In fact this growth factor may be presented in different ways with different epitopes exposed, thus making its detection difficult. For instance, as a function of pH, binding proteins, salt concentration or the presence of heparan sulfate, aFGF could modify its conformation (Wiedlocha *et al.*, 1992). We recently demonstrated that aFGF undergoes dimerization by interacting with the low affinity sites to form a three-component complex with the high affinity

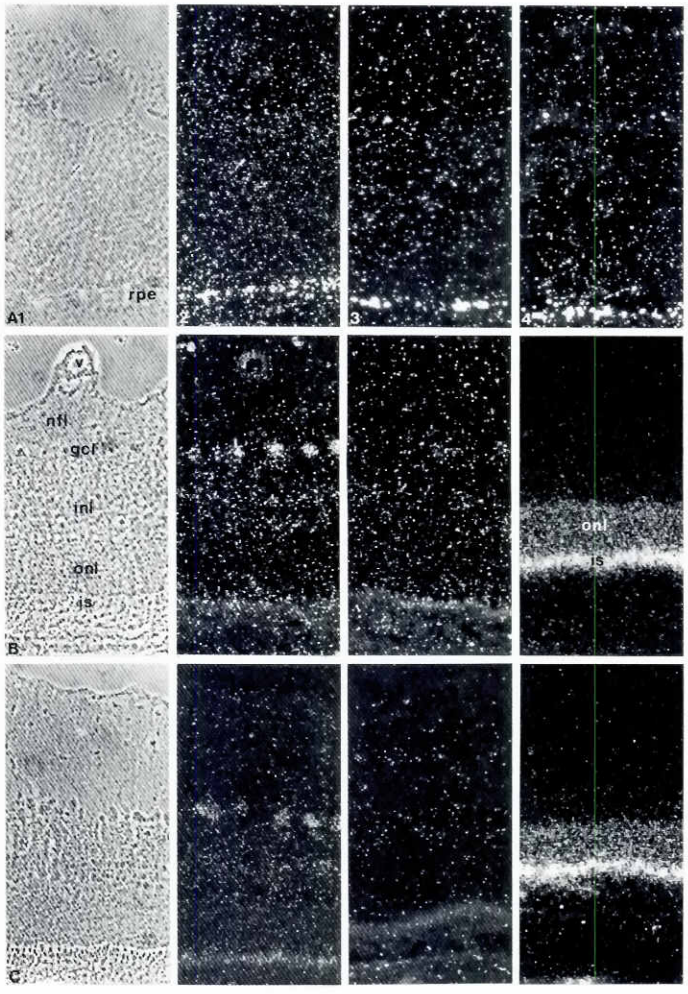

Fig. 2. aFGF and opsin *in situ* hybridization on developing retina. *Retina sections from 86-day-old calf embryo* **(A)**, *5-month-old calf* **(B)** *and 3-year-old cow* **(C)** *were hybridized with aFGF anti sense (1,2), a FGF sense (3) and opsin antisense (4). 1: phase contrast, 2,3,4: dark field. The labeling present with the aFGF antisense is distinct in calf* **(B)** *and cow* **(C)** *from the one obtained with aFGF sense. Opsin gene is only expressed after 5 months of development. Abbreviations gcl, inl,is, nfl, onl, rpe, as in Fig. 2; v, vessel. x150.*

receptors (Mascarelli e*t al.*, 1993). These different possible forms may result in different staining patterns depending on the antibodies used. Recent experiments performed in our laboratory on the rat retina using another batch of antibody raised against recombinant human aFGF but injected in the presence of heparin labeled more intensely the photoreceptor inner segments (Hicks, personal communication).

The *in situ* expression of mRNA using riboprobes from the coding region corroborates that the adult bovine retina is a relatively rich source of aFGF mRNA. We have shown recently that the aFGF protein is encoded by a message containing, in addition to the coding sequence, several untranslated exons which give rise to several transcripts (Philippe *et al.*, 1992). It would be interesting to study whether there is any specificity to the use of these exons either during development or in various cell types.

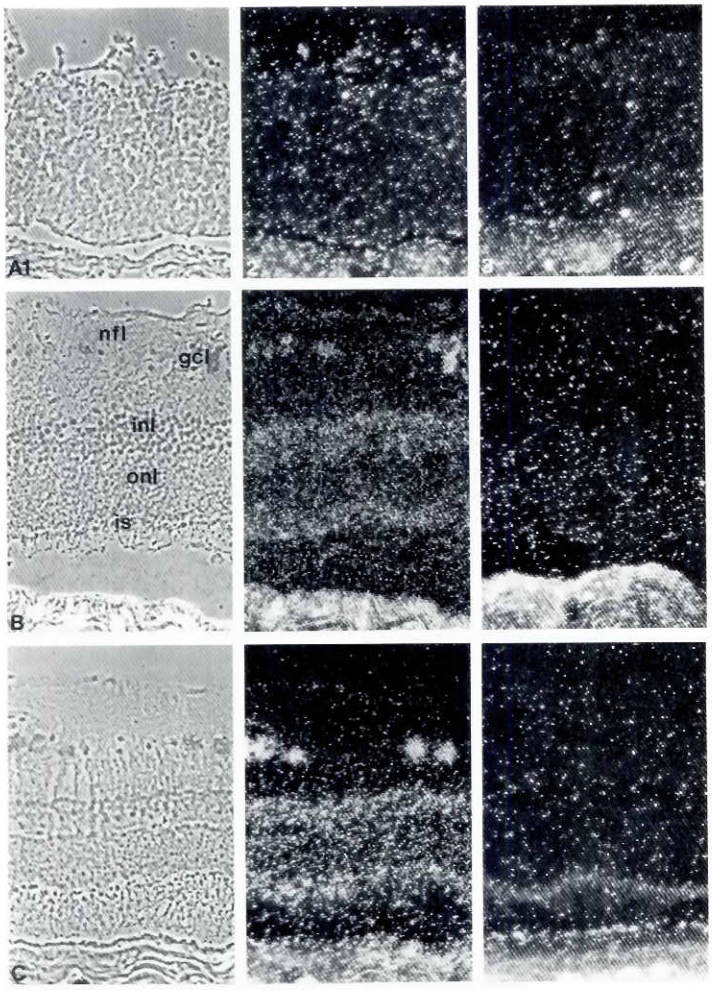

Fig. 3. aFGF *in situ* hybridization from embryo to old retina. *Retina sections from 4-5-month* **(A)**, *8-9-month-***(B)***-old calf embryos and 7-year-old bovine* **(C)** *were hybridized with a FGF antisense (1,2) or aFGF sense (control) (3). 1: phase contrast; 2,3: dark field. x110.*

In situ hybridization and immunocytochemistry reveal that all neuronal layers express aFGF with a timing corresponding to their sequential differentiation, starting with a high expression in the ganglion cells as already described (Jacquemin *et al.*, 1990; Noji *et al.*, 1990) and followed by the expression in the photoreceptor nuclei layer. This layer expressed less aFGF mRNA than other layers. Due to the low resolution of this technique, it is not possible to demonstrate whether subsets of neurons or glial cells also express aFGF differently *in vivo*. Glial cells in the nerve fiber layer which do not express aFGF mRNA are immunostained as shown previously for glial cells in the optic nerve (Faucheux *et al.*, 1991a) and the spinal cord (Tourbah *et al.*, 1991, 1992). RPE cells also express aFGF mRNA, but due to the autofluorescence of the pigments, it was difficult to demonstrate the presence of the protein by immunocytochemistry. No extracellular localization of aFGF could be demonstrated apart from in the vessel walls, where bFGF has already been found (Hanneken *et al.*, 1989; Gonzales *et al.*, 1990). These data can be compared with the results obtained on retinal cells in culture. *In vitro*, rat Müller cells express no aFGF mRNA even when assayed by PCR amplification (Bugra *et al.*, personal communication). RPE primary cell cultures and subcultures also express aFGF mRNA and protein (Malecaze *et al.*, 1993). It is noteworthy that FGF5 and bFGF mRNA have recently been detected *in vitro* in human RPE cells (Bost *et al.*, 1992). There is always the possibility that the expression observed *in vitro* is due to a reexpression of a normally silent gene. This is the case for the expression of GFAP, which is undetectable in adult bovine Müller cells and which is reexpressed in damaged retina *in vivo* or in cell culture (Mascarelli *et al.*, 1991). However, both *in vivo* and *in vitro* RPE cells synthesize aFGF (Jacquemin *et al.*, 1990; Malecaze *et al.*, 1993).

The autocrine or paracrine mechanism of action of FGFs is conventionally directed by their action on high and low affinity receptors. Among the different members of the FGF receptor family, FGF R1 and R2 mRNA have been described in the embryonic and adult retina (Heuer *et al.*, 1990; Tcheng *et al.*, personal communication). aFGF has a strong affinity for both receptors. Previous studies using ^{125}I FGF on the adult bovine retina (Faucheux *et al.*, 1991b) have demonstrated the presence of low and high affinity binding sites for aFGF and bFGF. Studies on the specificity of these sites indicated that they represent high and low affinity FGF receptors but could not distinguish between the different members of the family. By the same technique we could study their organization during chick (Cirillo *et al.*, 1990) and mouse (Jeanny *et al.*, 1987; Fayein *et al.*, 1990) retinal ontogeny. As for aFGF expression, the pattern of FGFRs follows the sequential differentiation of the neuronal layers starting with the ganglion layer and ending with the photoreceptor layer. Despite the fact that we have not examined all the intermediate stages of bovine development, the pattern appears similar. Thus, one could expect that aFGF induction during development is coordinated with the expression of its receptors and that it acts by an autocrine or paracrine pathway to modulate differentiation. In several studies, FGF has been implicated in mouse or rat retinal cell differentiation *in vitro* (Hicks and Courtois, 1988, 1992; Guillemot and Cepko, 1992), but does not seem involved in determination, although in some species, such as the chick embryo, FGF directly induces *in vivo* and *in vitro* the transdifferentiation of pigmented retinal cells to neural retina (Park and Hollenberg, 1989, 1991; Pittack *et al.*, 1991). The observation of the presence of the protein in the absence of detectable messages in the glial cells of the nerve fiber region is in favor of a paracrine route for these cells. The endogenous role for aFGF is not well understood. Many growth factors, including FGFs, have the properties of increasing the survival of cells *in vitro* and *in vivo* (Sievers *et al.*, 1987; Hicks and Courtois, 1988; Lipton *et al.*, 1988). The continuous expression of aFGF within retinal neurons in the adult may also be required for their own maintenance and not only for cell communication.

Materials and Methods

Retina were freshly dissected at the slaughterhouse (Vianor, Meaux, France) from calf embryos (86 days, 4-5 months, 8-9 months), calf (5 months) and cows (1,2,3,4,5,6,7 years). They were instantly mounted in Tissue Tek (OCT, Miles, Puteaux, France), frozen in liquid nitrogen and stored at -70°C. Cutting was done at -20°C first with a SLEE cryostat (London, UK) then with a Bright OTF/AS cryostat (DIS: Dhondt Instruments Scientifiques, Blanc-Mesnil, France). For in *situ* hybridization, 10 μm thick sections were collected on sterile slides coated with 0.5% gelatin and 0.05% chrome alum under RNAse free conditions. They were immediately fixed for 30 min in 4% paraformaldehyde in PBS. After washing in 3xPBS (10 min) and dehydration in ethanol 70% (5 min) and 95% (2x5 min), the slides were stored at -20°C. On the day of hybridization, sections were rehydrated in 95,

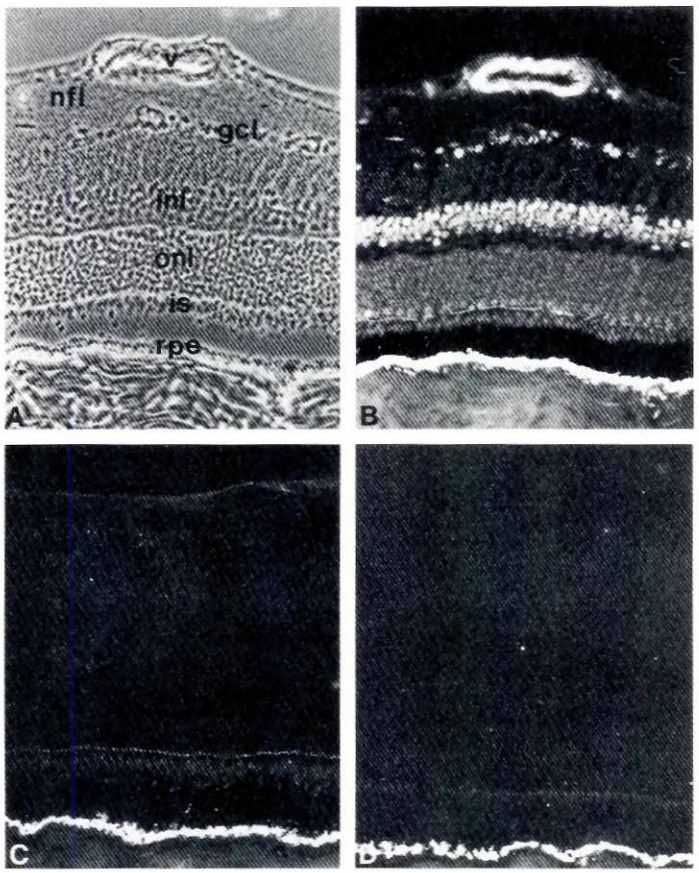

Fig. 4. aFGF immunodetection in 3-year-old bovine retina. (A,B) *Anti-aFGF serum;* **(C)** *preimmune serum;* **(D)** *phosphate buffered saline (PBS).* **(A)** *Phase contrast;* **(B, C, D)** *fluorescence. The immunohistochemistry simple staining procedure reveals a diffuse intranuclear localization of aFGF in all neuronal layers. A staining is also visible on the blood vessels and on glial cells of the nerve fiber layer. The use of preimmune serum or PBS induces no fluorescence. x140.*

85, 70, 50 and 30% ethanol (5 min each) containing 0.3 M ammonium acetate, rinsed in PBS (5 min), treated with 0.02 M HCl (10 min), 0.01% Triton X-100 in PBS (1.5 min), 1 µg/ml proteinase K in 50 mM Tris, 5 mM EDTA pH 7.4 (7.5 min), rinsed in PBS/glycine (2 mg/ml), dehydrated through a graded ethanol series and air dried. A 20 µl aliquot of probe (specific activity: 1-1.5x10^8 cpm/µg) was deposited on each slide and spread over the sections with siliconized glass coverslip.

The aFGF probe used was a Pst I/Xba I fragment of 1145 bp obtained from a bovine aFGF cDNA clone (Alterio *et al.*, 1988; Halley *et al.*, 1988) and included 293 bp of the coding region. It showed 63% homology in the coding region with bFGF and hybridized specifically by Northern analysis to a major aFGF transcript of 4.2 kb. The antisense and sense riboprobes subcloned into bluescript (Stratagene) were respectively labeled by transcription with T7 and T3 polymerase and ^{35}S-r UTP (specific activity: >1000 Ci/m mol) (Amersham, Les Ulis, France). They were reduced in size (200 bases) by alkaline hydrolysis and diluted (10^7 cpm/ml) in hybridization buffer containing 50% formamide, 750 mM NaCl, 25 mM Pipes, 25 mM EDTA, 1x Denhardt's, 100 mM DTT, 0.2% SDS, 250 µg/ml DNA from salmon sperm, 250 µg/ml poly A, 5% dextran sulfate. The procedure of hybridization was largely based on Simmons *et al.* (1989). Slides were incubated overnight at 42°C in a humidified atmosphere. Coverslips were then removed by immersing the slides in 4xSSC. Slides were washed in 4xSSC (5 min) before RNAse A digestion (20 µg/ml in 10 mM Tris, 500 mM NaCl, 1 mM EDTA) at 37°C for 30 min to remove unhybridized RNA probe. After washing in 2xSSC, 1 mM DTT (2x5 min) then in 1XSSC, 1 m M DTT (10 min), and in 0.5xSSC, 1 m M DTT (10 min) at room temperature, they were treated at 55°C for 30 min in 0.1xSSC, 1 m M DTT, then washed quickly in cold 0.1xSSC, 1 mM DTT before dehydration with ethanol 50, 70, 95 and 100% (3 min each). Autoradiography was performed by dipping the air dried slides into LM1 (Amersham, Les Ulis, France) or K5 (Ilford, Saint Priest, France) emulsions. After 4 weeks exposure at 4°C, slides were developed in Kodak D19 and fixed in Hypam Ilford fixer. Photographs were taken on a photomicroscope (Zeiss, Oberkochen, Germany) or on an Aristoplan (Leitz, Wetzlar, Germany) using PAN F film (Ilford).

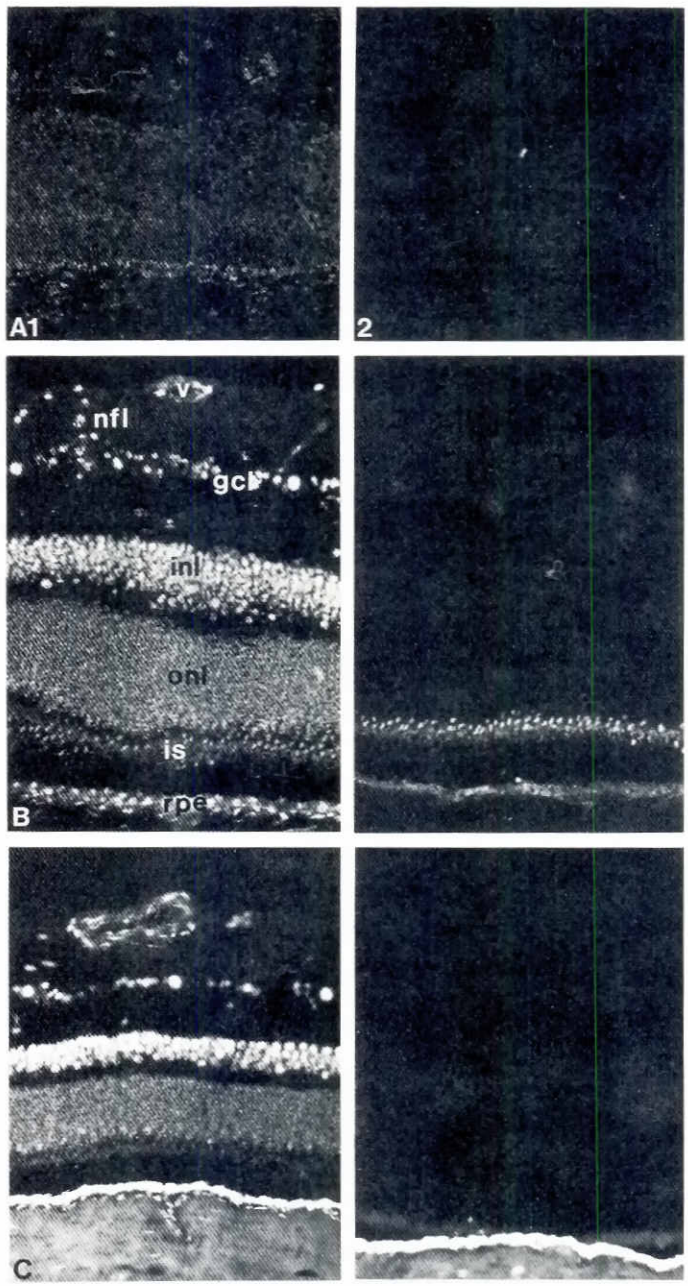

Fig. 5. Indirect immunofluorescence staining of aFGF on developing retina. (A) *86-day-old calf embryo;* **(B)** *5-month-old calf;* **(C)** *3-year-old cow. 1: Affinity purified anti aFGF IgG; 2: non retained fraction from the aFGF Aminolink column. The immunostaining is visible in calf and cow retina. Controls with nonretained fraction are negative. x140.*

Opsin *in situ* hybridization was performed under identical conditions using the bovine cDNA clone from Dr. Barnstable (Treisman *et al.*, 1988) and already used in adult and regenerating newt retina (Bugra *et al.*, 1992).

For antibody labeling studies, the same stages of the embryonic and post-natal development were considered. Unfixed tissues were cut at -20°C with a cryostat. Sections of 10 µm were collected on slides coated with gelatin and chrome alum. After fixation (4% paraformaldehyde, 30 min), permeabilization (0.1% Triton X-100) and saturation (PBS, 1% BSA, 1% normal goat serum), the sections were incubated for 1 h at 20°C with a 1/100 dilution of the different fractions of antibodies.

Polyclonal antibodies were prepared in two different rabbits against recombinant human aFGF purified to homogeneity from bacterial cell lysates by heparin Sepharose chromatography and analyzed by HPLC. IgG fractions were prepared from the immune serum by DEAE Sepharose chromatography. Purified anti aFGF antibodies were obtained by affinity chromatography of the IgG fraction passed through an aFGF Aminolink column. The specificity of the aFGF antibodies was previously determined by ELISA and Western blotting (Oliver *et al.*, 1992). The IgG not retained from the aFGF Aminolink column did not contain any detectable anti aFGF antibodies. The retained IgG fraction did not recognize bFGF but bound a 17 kDa band corresponding to aFGF. After the first incubation, slides were rinsed, then incubated with a biotinylated donkey anti-rabbit IgG (Amersham) 1/100, then with fluorescein isothiocyanate (FITC)-labeled extravidin diluted 1/100 (Sigma, St Quentin Fallavier, France). All incubations were followed by 6 washes of 5 min in PBS. Finally, the slides were mounted in glycerol-PBS (1/1) and examined using a Zeiss photomicroscope or a Leitz Aristoplan equipped with an epifluorescence illumination with a HBO 50W lamp. Photographs were taken with Ilford HP5 film (400 ASA).

Control experiments were performed by 1) omitting one of the antibodies of the first incubation, 2) replacing the latter by PBS, 3) using the preimmune serum of the rabbit, 4) using an aFGF preabsorbed antibody, and 5) using the non-retained fraction of the affinity aFGF Sepharose column.

To localize astrocytes and vascular endothelial cells, sections were incubated with polyclonal rabbit anti GFAP and Von Willebrandt factor (Dako, Trappes, France) respectively.

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