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Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGFα

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Summary

Proliferation in the rat retina, as in other parts of the nervous system, occurs during a restricted period of development. In addition to regulating cell number, the mechanisms that control proliferation influence the patterning of tissues, and may affect the determination of cell type. To begin to determine how proliferation is controlled, several growth factors found in the retina were tested for effects on progenitor cell division in culture. Proliferation was enhanced by TGFα, bFGF and aFGF, and many of the dividing cells later differentiated into cells with the antigenic phenotypes of retinal neurons and glial cells. The mitotic response of retinal cells to these factors changed during development: progenitor cells from younger retinas (embryonic day 15 to 18; E15-E18) were more responsive to FGF's, while progenitor cells from older retinas (>E20) were more responsive to TGFα. Progenitor cells stopped dividing in vitro, even when treated with excess mitogen. These observations suggest that proliferation in the retina may be stimulated by multiple mitogenic signals provided by TGFα, FGF, or related factors, and that proliferation is not controlled by limiting concentrations of mitogen alone. Rather, these data demonstrate that retinal cells change during development in their responsiveness to mitogenic signals. Such changes may contribute to the regulation of proliferation.

Key words: retina, TGFα, FGF, proliferation.

Introduction

The number of cells in the rodent retina increases exponentially during embryonic development; proliferation declines, however, around the time of birth, and stops approximately 1 week later (Denham, 1967; Young, 1985; M. LaVail, D. Yasumura, and D.H. Rapaport, personal communication). At characteristic times throughout embryonic and postnatal development, multipotential progenitor cells produce postmitotic progeny, which develop into the seven major classses of retinal cells (Young, 1985; Turner, et al., 1990; M. LaVail, D. Yasumura, and D.H. Rapaport, personal communication). At least some newly generated postmitotic cells begin to differentiate while still in the "mitotic zone" at the ventricular surface of the retina, prior to migrating inward where they will form the laminae characteristic of the mature retina (Hinds and Hinds, 1974; Barnstable et al., 1985; McLoon and Barnes, 1989). The mitotic zone of the retina thus comprises a mosaic of mitotic cells and postmitotic differentiating cells; proliferation therefore must be regulated in such a way as to permit some cells to become postmitotic while neighboring cells continue to divide.

In principle, the proliferation of retinal progenitor cells could be controlled in several ways. An intrinsic program wherein cell divisions are in some way "counted", as suggested for O-2A progenitor cells (Temple and Raff, 1986), could be the underlying control mechanism. Several observations from studies of cell lineage in the retina, however, are not easily explained by an intrinsic counting mechanism. Clones from retinal progenitor cells labeled with a lineage marker at early embryonic stages (e.g., E13-14) exhibit a wide range of sizes, comprising clones of from 1 to 234 cells (Turner et al., 1990). Furthermore, the presence of early and late generated cells types in the same clone suggests that clonally related cells do not stop dividing synchronously. Although these observations could reflect a great heterogeneity in the intrinsic proliferative capacity of retinal progenitor cells, they could also reflect an extreme influence of the environment in which each daughter of a cell division independently assesses the positive and/or negative cues in its environment that regulate cell division.

The role of intrinsic and environmental regulatory mechanisms in the control of proliferation in the retina was addressed in a recent study of rat retinal cells in vitro. Data from this study indicated that embryonic and postnatal progenitor cells behaved differently in the same environment: postnatal progenitor cells exposed to an embryonic environment did not divide as extensively as embryonic progenitor cells, and embryonic progenitor cells exposed to a postnatal environment continued to divide more than postnatal progeni-

tor cells (Watanabe and Raff, 1990). While these observations do not preclude changes in the levels of mitogenic or inhibitory signals with age, they are not easily explained by changes in the environment alone; rather, they suggest that the proliferative potential of progenitor cells changes during development, perhaps reflecting changes in the responsiveness of progenitor cells to environmental signals. In order to determine the contribution of changes in progenitor cells and changes in their environment to the control of proliferation, it is first necessary to identify environmental signals that influence the proliferation of retinal progenitor cells.

As a first step toward identifying the signals that may regulate proliferation in vivo, we have defined factors that influence proliferation in vitro. A number of well characterized growth factors have been shown to be expressed in the retina and the surrounding pigment epithelium; these include bFGF (Nyberg et al., 1990), int-2 (Wilkinson, et al., 1989), NGF (Chakrabarti et al., 1990), insulin (Meimaridis et al., 1990), TGFβ_2 (Millan et al., 1991), and TGFα (Anchan et al., 1991). Receptors for some of these factors have also been identified in the retina (Anchan et al., 1991; Yan and Johnson, 1988; Wanaka et al., 1991). In this study we assayed several of these factors for effects on proliferation in cultures of embryonic and postnatal rat retinal cells. Having identified factors that stimulate proliferation, we determined that responsiveness to these mitogenic signals changes during development.

Materials and methods

Preparation of cultures

Retinas from embryonic and postnatal Sprague-Dawley rats were dissected free of surrounding ocular tissue, and collected on ice in L-15 medium (in some experiments, Long-Evans rats were used instead, and no significant differences were observed). The age of embryonic rats was determined from crown-rump length and examination of external features (Angulo Y Gonzales, 1932; Long and Burlingame, 1938). In most experiments, newborn retinas were taken from animals within 6 hours of birth. When feasible (i.e , when the retinas were large enough), peripheral, rather than whole, retina was used to minimize the number of endothelial cells and astrocytes in the cultures: these cells are found predominantly in the central part of the retina at the ages used (Watanabe and Raff, 1988). A strip of peripheral retina approximately 1-2 mm wide was cut away and transferred to another dish containing L-15 medium where it was cut into pieces 0.5-0.75 mm^2. Cultures of E15-16 retinal cells were prepared from whole retina, however. For explant cultures, five pieces of retina were either transferred to a nucleopore filter (0.2 μm pore) floating in a 35 mm dish in 1.5 ml of culture medium (see below) or placed in agarose-coated (1% NuSieve) wells of a 24-well plate in 0.5 ml of medium (agarose prevents attachment of explants to the wells). Similar results were obtained with both methods. For monolayer cultures, pieces of peripheral retina were treated with trypsin (0 1%, Sigma) in $Ca^{2+}Mg^{2+}$-free Earles buffer for 20 min at 37°C. The tissue was triturated with a Pasteur piptette in the presence of DNase (0.1 mg/ml, Sigma) and egg white trypsin inhibitor (0.1 mg/ml, Sigma) and washed once with culture medium. Unless otherwise indicated, cells were plated at a density of 50,000 cells per well on 12 mm glass coverslips that had been pretreated overnight with polyornithine (125 μg/ml) and coated with laminin (20 μg/ml, Collaborative Research) for 4-6 hours. Cells were grown in 0.5 ml of DMEM/F12 (1:1) containing insulin (5 μg/ml), transferrin (human, 100 μg/ml), selenium (0.04 ng/ml), putrescine (16 μg/ml), tri-iodothyronine (0.3 ng/ml), thyroxine (0 4 ng/ml) and progesterone (0.06 ng/ml) (Bottenstein and Sato, 1979). TGFα (human recombinant, Amgen), bFGF (human recombinant, Amgen), aFGF (bovine brain, R&D), EGF (human recombinant, Amgen), PDGF (human recombinant type AA, Collaborative Research), NGF (B subunit, University of Michigan-Flint), and TGFβ_2 (porcine platelet, R&D) were added daily Growth factors were prepared according to manufacturers' instructions and stored as concentrated stocks (100 μg/ml) at −70°C. They were diluted 10-fold in L-15 medium for short term storage (i.e , less than 1 week) at 4°C.

Assessment of proliferation

Monolayers

Because there are no markers available to distinguish retinal progenitor cells from other types of retinal cells, and because the proportion of progenitor cells varies in retinas of different ages, the progenitor cells in starting populations were identified by their capacity to divide, monitored by incorporation of bromodeoxyuridine (BrdU; 4 μM, Boehringer), which was added at the time cells were plated. To determine how many of the original progenitor cells were still dividing after 2-3 days in vitro, cultures were exposed to [^{3}H]thymidine ([^{3}H]thy; 0.5 μCi/ml, 80 Ci/mM, NEN) for 24 hours on the last day in culture. BrdU was visualized immunocytochemically and [^{3}H]thy incorporation was determined following autoradiography. coverslips were coated with emulsion (Kodak NTB2), exposed for 2 days, and developed in D-19 (Kodak). The proportion of BrdU-labeled cells that were also labeled with silver grains was determined by counting 300-400 cells per coverslip Nearly all [^{3}H]thy-labeled cells were also BrdU$^+$ In most experiments, each condition was assayed in triplicate and each experiment was repeated at least once. Results from 2-4 experiments have been pooled and are expressed as the mean ± s.e m. Staining with BrdU revealed the size of the nuclei, while most of the dividing cells had small nuclei, some cells with larger nuclei were also labeled with BrdU (for example, see Fig. 5), and these increased in number after 3 days in culture. Cells with larger nuclei were not included in the counts of dividing retinal progenitor cells· they appeared to be induced to divide as a result of dissociation, as they were not labeled with BrdU if explants, rather than monolayers, were exposed to BrdU, though they subsequently incorporated [^{3}H]thy after dissociation and growth in monolayer culture. This population of cells may include non-retinal cells (e.g , fibroblast-like cells) and some Muller cells, which do not divide in vivo under normal circumstances (Hicks and Courtois, 1990).

Explants

Tissue was treated with BrdU and [^{3}H]thy as for monolayers; to facilitate counting, cells in explants were dissociated with trypsin after three days in vitro, allowed to attach to polylysine-coated glass cover slips for 30-60 min and processed for immunocytochemistry and autoradiography as for monolayer cultures. In some cases, explants were only exposed to BrdU for 3 hours after 3 days in culture before trypsinization and processing for BrdU immunohistochemistry.

In vivo

To estimate proliferation in vivo, retinas were removed from embryonic and postnatal rats and exposed to BrdU (10 μM) for 3 hours in a dish of culture medium to permit equal access to BrdU at all ages tested. To confirm that this accurately reflects proliferation in vivo, results obtained from this procedure were compared to BrdU incorporation following injection of newborn rats (intraperitoneal, 3 times over 3 hours, 0.1-0.2 μg/g body weight). Similar results were obtained: 26.1 ± 2.1% of the retinal cells divided in a dish compared to 26 3 ± 1.2% after injection in vivo.

Immunocytochemistry

Cultures were fixed in 4% paraformaldehye in 0.1 M phosphate buffer, pH 7 4 for 1-2 minutes. Before staining with anti-BrdU antibody (1 hour at room temperature, culture supernatant diluted 1·1 in PBS containing 0.5% triton-X 100 and 2% FCS), cells were treated with acid-alcohol (−20°C), followed by exposure to 2N HCl (20 min) and 0 1 M borate buffer, pH 8.5 (5 min) (Yong and Kim, 1987). Labeling of BrdU was visualized with Texas Red horse anti-mouse IgG (1:100; Jackson Laboratories). Cultures were then dried and processed for autoradiography, or mounted in gelvatol and viewed with a Zeiss Axiophot microscope For staining with markers that distinguish different types of retinal cells, cultures were stained with the antibodies 115A10 (ascites fluid, 1:50; Onoda and Fujita, 1987), Ret B1 (ascites fluid, 1·100, Barnstable et al , 1983), HPC-1 (ascites fluid, 1:1000; Barnstable et al , 1985), VC1.1 (ascites fluid, 1.100, Arimatsu et al., 1987), Ret P1 (ascites fluid, 1:100; Akagawa and Barnstable, 1986), RetG1 and RetG2 (both at 1:100; Barnstable, 1980), and rabbit anti-GFAP (1:1000; Pruss, 1979) for 30 min at room temperature, followed by either Texas Red horse anti-mouse IgG or fluorescein goat anti-rabbit antisera (both 1:100, Jackson Laboratories). Cultures were then processed for staining with BrdU antibody, which was visualized with either Texas Red or fluorescein horse anti-mouse IgG (1 100, Jackson Laboratories).

TGFα immunohistochemistry

E16.5-P5 retinas were fixed in 1% paraformaldehye in 0.1 M phosphate buffer, dehydrated, and embedded in paraffin. 6 μm sections were mounted on gelatin-coated slides, rehydrated and stained according to manufacturer's instructions (except that sections were treated with trypsin for only 2.5 min), using TGFα antibody at 5 μg/ml (Oncogene Science, Ab 2) Staining was visualized with biotinylated anti-mouse IgG (Vector) followed by avidin-biotin HRP (Vectastain), using diaminobenzidine as the chromogen Specifity of staining was confirmed by blocking with excess TGFα (50 μg/ml); the same concentration of EGF did not effect staining.

Results

Retina produces mitogen(s) in vitro

Proliferation of retinal progenitor cells has been shown to be maintained at high levels when retinal cells are cultured as explants or aggregates (Vollmer and Layer, 1986; Sparrow et al., 1990; Watanabe and Raff, 1990). In most of these studies, retinal cells were grown in the presence of serum, which contains a variety of mitogens. Thus, it was not clear whether the serum or the retina was the source of mitogenic signals. To clarify this issue, explants of rat retina were cultured in serum-free medium. Pieces of peripheral retina were used for these studies, rather than whole retina, to reduce the number of non-retinal cells in the cultures: at the ages studied, most non-retinal cells, e.g., astrocytes and endothelial cells, are concentrated in the central portion of the retina (Watanabe and Raff, 1988). The retinal identity of dividing cells was also confirmed using antibodies that label differentiated retinal cells (see below). Proliferation was assessed in two ways: the proportion of total cells that were still dividing after 3 days in culture was determined by exposing cultures to the thymidine analog BrdU during the last 3 hours of culture. This facilitated comparison of proliferation in vitro to proliferation at equivalent ages in vivo. It was also necessary to compare proliferation at different times during development, when the proportion of progenitor cells in the starting population varies considerably. To do this, proliferation after three days in culture was normalized to the initial population of mitotic cells: cells that entered the cultures as dividing cells were identified by their ability to incorporate BrdU, introduced at the beginning of the culture period; the proportion of these cells that were still mitotic after 2-3 days in vitro was determined by adding [^{3}H]thymidine ([^{3}H]thy) during the last day of culture. After staining with an antibody to BrdU and processing for autoradiography, the proportion of BrdU$^+$ cells that were also labeled with [^{3}H]thy was counted (see Fig. 3 for examples of BrdU$^+$, [^{3}H]thy labeled cells).

Using these methods, many retinal cells in explants were found to divide for several days in the absence of serum, and the level of proliferation was similar to that observed in explants grown in 10% fetal calf serum (FCS). For example, in cultures of E17-E18 retinal explants, 29.5 ± 3.7% of the cells continued to divide after 3 days in vitro in the absence of serum, compared to 35.7 ± 4.5% in explants grown in serum. The serum-free medium used for these studies contained a high concentration of insulin (5 μg/ml), which can activate IGF-1 receptors (McMorris et al., 1986). Reducing the concentration of insulin in explant cultures of embryonic retina did not effect proliferation (not shown), indicating that proliferation seen in explants was not stimulated by high exogenous levels of insulin.

Comparison of proliferation in explants of retinas from different ages grown in serum-free medium showed that proliferation in vitro declined in parallel with the decline seen in vivo, though the level of proliferation in vitro was always lower than that seen in vivo at equivalent ages, by approximately 2-fold (Fig. 1). These observations suggest that retinal tissue continues to produce mitogen(s) in vitro for at least several days. As in vivo, a higher proportion of cells divide in explants of embryonic retina than in explants of early postnatal retina.

FGF and TGFα enhance proliferation in explant cultures of perinatal retina

To begin to identify factors that stimulate proliferation in retinal cultures, explants were grown in the presence of several of the peptide growth factors reported to be

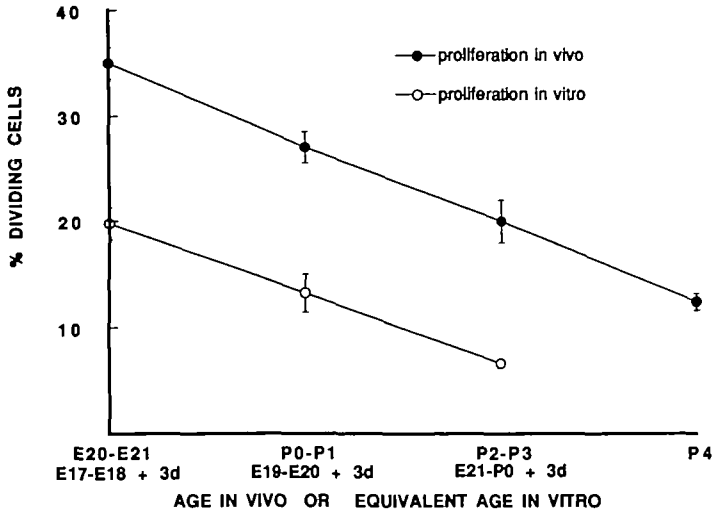

Fig. 1. Comparison of proliferation in vivo and in explant cultures. The proportion of total retinal cells that incorporate BrdU during a 3 hours pulse was determined after retinal explants had been maintained in culture in serum-free medium for 3 days (open circles), or at equivalent ages in vivo (closed circles). E20-21, etc. refer to age in vivo; E17-18 + 3, etc. refer to age in vitro.

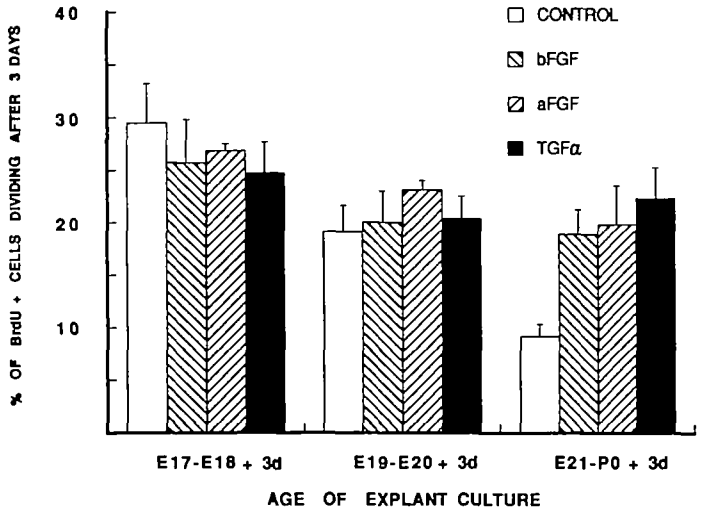

Fig. 2. Proliferation in explant cultures treated with growth factors. The proportion of retinal progenitor cells (BrdU labeled at start of culture period) that continued to divide after 3 days in culture was determined by adding [^{3}H]thymidine for the last 24 hours of the culture period. Explants were prepared from retinas at the ages indicated and treated with concentrations of growth factors found to be saturating at those ages in monolayer cultures (see Figs 6 and 7).

present in the retina. In these and all subsequent experiments, proliferation was assessed by the BrdU/[^{3}H]thy double-labeling method; these results are therefore not directly comparable to data presented in Fig. 1. As shown in Fig. 2, proliferation in explants of late prenatal and early postnatal retina was enhanced by treatment with acidic or basic fibroblast growth factors (aFGF or bFGF), as well as transforming growth factor alpha (TGFα). By contrast, treating explants of younger retina (E17-E20) with these factors did not significantly alter the level of proliferation (Fig. 2). This lack of effect could be explained in several ways: younger progenitor cells may be unresponsive to these factors, for example, or explants of younger retina may contain saturating levels of endogenous mitogenic or inhibitory signals.

FGFs and TGFα enhance proliferation in monolayer cultures

To further analyze proliferation of retinal progenitor cells, an effort was made to reduce signaling among cells by dissociating retinal cells and culturing them as a relatively sparse monolayer. While initially plated as single cells, small, flattened clusters of cells formed over 2-3 days, a result of both division and aggregation, as clusters contained both mitotic (BrdU$^+$, or BrdU$^+$, [^{3}H]thy$^+$) and postmitotic (BrdU$^-$, [^{3}H]thy$^-$) cells (Fig. 3).

Proliferation appeared to depend on cell number and/or cell density in monolayer cultures. When cells were cultured at a starting density of approximately 50,000 cells per 16 mm well, proliferation of cells from E17 retinas was significantly reduced compared to explants prepared from the same tissue (Table 1). In monolayer cultures of E17 cells plated with 4-fold more cells (200,000 cells per well), an intermediate level of proliferation was observed (Table 1). Proliferation in monolayers of older (E21 and P0) retinal cells was even further reduced, relative to explants of the same tissue (Table 1). This dependence of proliferation on increasing numbers or density of retinal cells suggests that it involves signaling among cells rather than autocrine mechanisms. It is not yet clear, however, whether direct contact between cells is required for this signaling, as suggested for cerebellar progenitor cells (Gao et al., 1991).

Addition of aFGF, bFGF, or TGFα to monolayer cultures restored proliferation to the levels seen in

Table 1. *Density-dependence of proliferation*

Age	Type of culture	% BrdU$^+$ cells dividing after 3 days
E17	Explant	36±6.9
	Monolayer - 50,000	10 5±1 4
	Monolayer - 200,000	17 2±2 9
E21	Explant	12 1±1 3
	Monolayer - 50,000	1 0±0 2
	Monolayer - 200,000	2 0±0 5
P0	Explant	7 2±1 5
	Monolayer - 50,000	0 4±0.1
	Monolayer - 200,000	0 1±0 1

Explants or monolayer cultures were prepared from retinas at the indicated age and grown in serum-free medium for 3 days. Proliferation was assessed by double-labeling with BrdU and [^{3}H]thymidine as described Monolayer cultures were plated at starting densities of either 50,000 cells or 200,000 cells per 16 mm well.

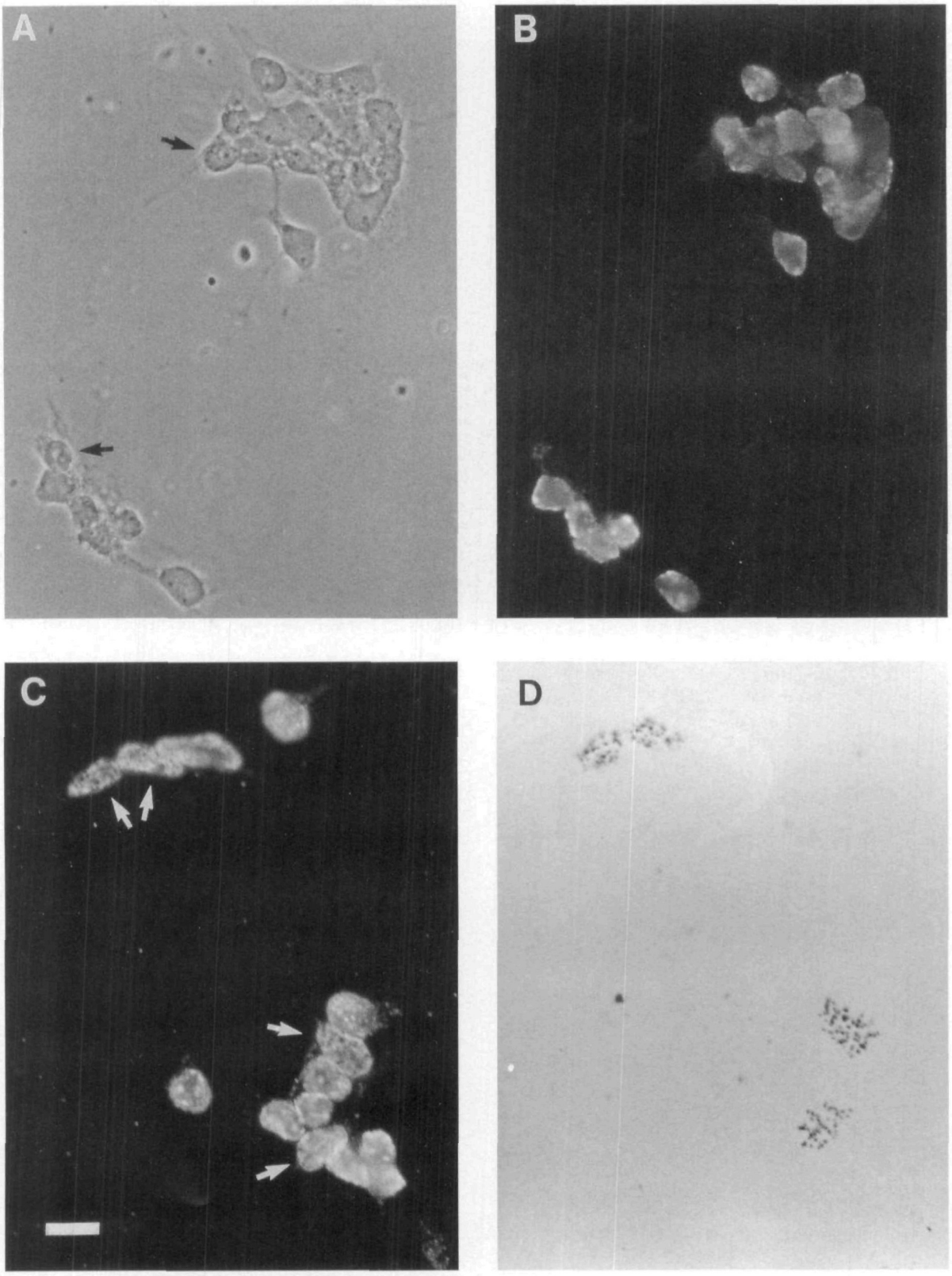

Fig. 3. Progenitor cells divide after 3 days in vitro. Monolayer culture of E18 cells (grown in bFGF, 1 ng/ml for 3 days) show small, flattened clusters containing BrdU-positive retinal progenitor cells (panels B and C, texas red). The clusters shown in B also contain BrdU-negative cells (arrows in panel A, phase contrast). Four of the BrdU-positive cells in panel C (arrows) were still dividing after 3 days in culture (panel B; bright-field illumination shows overlying silver grains, indicating incorporation of [^{3}H]thymidine). Bar, 10 μm.

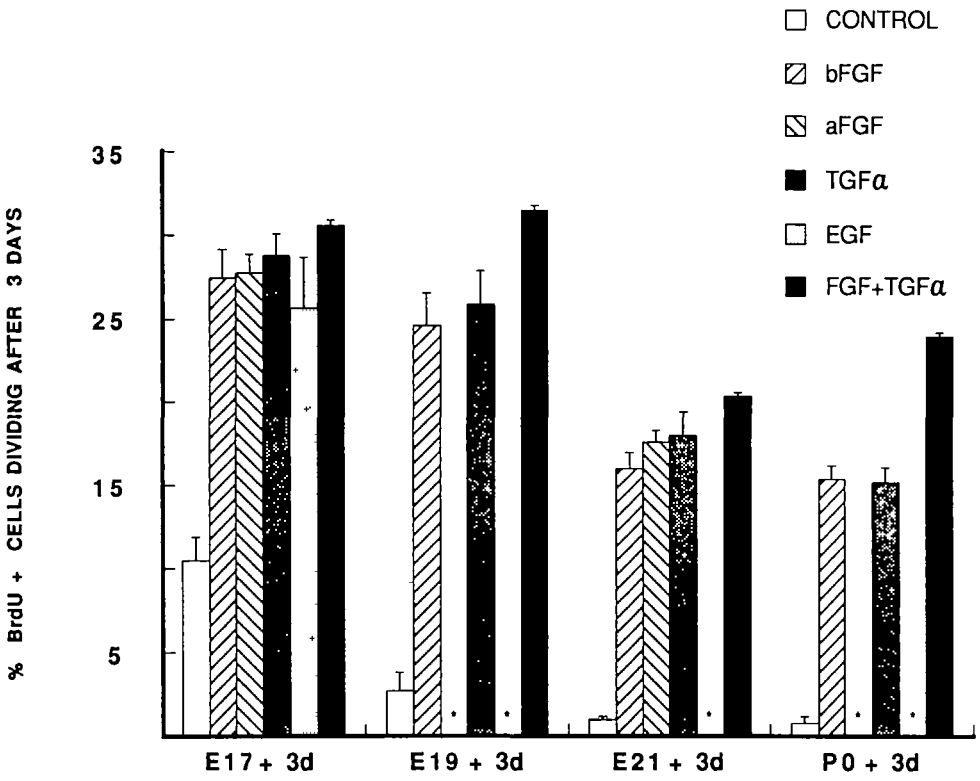

Fig. 4. Proliferation in monolayer cultures of retinal cells treated with growth factors. Cells were cultured at a starting density of 50,000 cells per 16 mm well and the proportion of progenitor cells (BrdU labeled at start) that continued to divide ([^{3}H]thymidine labeled) after 3 days in culture was determined. Cells were treated with concentrations of growth factors determined to be saturating for the indicated ages (see Figs 6 and 7). EGF was used at a concentration of 50 ng/ml. (*) not determined.

explants (Fig. 4). Proliferation was also enhanced by EGF (Fig. 4); similar effects of TGFα and EGF on retinal cells were recently reported by Anchan, et al. (1991). Treatment with several other peptide growth factors, PDGF-AA (10 ng/ml), TGFβ_2 (10 ng/ml), and NGF (100 ng/ml), did not stimulate proliferation in cultures of E18 and E20 retina, nor did the combination of FGF and NGF increase the level of proliferation beyond that seen in FGF alone in E20 cultures (not shown), unlike the effect of this combination of growth factors on neural progenitor cells in other parts of the CNS (Cattaneo and McKay, 1990).

In monolayer cultures of embryonic retina, the effects of TGFα and FGFs were not additive, suggesting that they act on the same population of cells (Fig. 4). In postnatal cultures, the combination of these factors elicited greater responses than either factor alone (Fig. 4); this may indicate that distinct subpopulations of progenitor cells develop over time, or that some cells come to require a combination of both signals to divide optimally.

Retinal cells from younger embryos clearly respond to these growth factors when cultured as monolayers, although they did not appear to respond when cultured as explants. One explanation for this discrepancy is that retinal explants produce saturating levels of endogenous mitogen(s). To determine whether these "endogenous" mitogenic signals were readily diffusible, explants of E18 retina were added to monolayer cultures of E18 or P0 retinal cells. These explants did not stimulate nearby cells in the monolayer to divide (not shown). This suggests that the endogenous mitogenic signals may normally be bound to cell surfaces or the extracellular matrix and may not be freely diffusible.

Table 2. *Survival in monolayer cultures*

Experiment 1 E21	3 hours	1 day	3 days
Postmitotic ($BrdU^-$)	ND	757±25	653±46
Progenitor ($BrdU^+$)	ND	731±48	954±56
Total cells	1219±35	1488±23	1608±61
Experiment 2 E20		1 day	3 days
Postmitotic ($BrdU^-$)		517±36	526±59
Progenitor ($BrdU^+$)		923±138	957±67
Total cells		1440±171	1403±122

To analyze survival in the absence of growth factors, the number of cells in a diameter strip of the coverslip was counted using phase optics 3 hours after cells were plated, or 1 and 3 days later Cultures were exposed to BrdU when plated, and were fixed prior to counting 1 and 3 day-old cultures were stained with anti-BrdU antibody to distinguish postmitotic cells ($BrdU^-$) and progenitor cells (incorporated BrdU during first day in vitro) in the starting populations

Under the conditions used in these experiments, there was minimal loss of cells in the absence of growth factors (Table 2); in some experiments there was even a small increase in cell number in the absence of exogenous growth factors (Table 2, Experiment 1).

Addition of growth factors therefore appears to stimulate proliferation, rather than enhance survival. Survival was reduced, however, under several conditions: (1) if the low density cultures were maintained for more than 3 days in vitro, (2) if low density cultures were prepared from retinas older than P1, or (3) if the concentration of insulin in monolayer cultures was reduced from 5 μg/ml to 50 ng/ml, a level that would not be expected to significantly activate IGF-1 receptors (McMorris et al., 1986). Growth of E 18 cells in 50 ng/ml insulin resulted in a loss of cells that was apparent by inspection, and a 2-3-fold reduction in the level of proliferation in monolayer cultures not treated with growth factors (not shown). The reduction in proliferation could be interpreted in several ways: it could reflect the reduction in cell number/density, consistent with observations presented in Table 1; in this case, insulin/IGF-1 would be acting in vitro as a survival factor, as reported in cultures of neuroepithelial cells (Drago et al., 1991). Alternatively, it could indicate that IGF-1 or high levels of insulin contribute to the endogenous mitogenic signal. The effect of lowering insulin in monolayer cultures differs from that observed in explant cultures: in explants, reducing insulin did not affect proliferation. This could be due to higher endogenous levels of insulin or IGF-1 in the explants than in the monolayers.

Dividing cells are retinal progenitor cells

To confirm that cells that divided in response to addition of FGFs and TGFα were retinal progenitor cells, dividing cells were labeled with BrdU after 2 days in culture, and cultured for an additional 5 days to permit expression of differentiation markers that distinguish several populations of mature retinal cells. For these experiments, higher density cultures (e.g., 100,000-200,000 cells per well) prepared from newborn retina were used because survival for the extended period of time required for differentiation was better in higher density cultures. Proliferation in these cultures was also enhanced by FGFs and TGFα (Fig. 4), and proliferation due to endogenous mitogen was negligible

Table 3. *Fate of dividing cells*

Cell type (marker)	Mitogen	% $BrdU^+$ cells expressing marker
Bipolar (115A10)	TGFα	39.5±2.4
	bFGF	23.7±5.4
Bipolar (Ret B1)	TGFα	41.0±2.0
	bFGF	39.5±9.6
Amacrine (HPC-1)	TGFα	9
	bFGF	8
Amacrine (VC1.1)	TGFα	3.5
	bFGF	4.0
Muller (Ret G1+Ret G2)	TGFα	9.0±2.0
	bFGF	17.5±1.5
Rod (Ret P1)	TGFα	0
	bFGF	0
Astrocyte (GFAP)	TGFα	0.3±0.3
	bFGF	0

Newborn retinal cells, plated at 100,000 cells per well, were exposed to BrdU after 2 days in TGFα (1 ng/ml) or bFGF (100 ng/ml). After a total of 7 days in culture, cells were stained with the indicated antibodies and anti-BrdU. The proportion of cells with small $BrdU^+$ nuclei (see Fig. 5) that also expressed differentiation markers was determined.

(Table 1). Many of the cells that divided ($BrdU^+$) in response to these factors expressed markers reported to label bipolar (115A10 and Ret B1; Onoda and Fujita, 1987; Greferath et al., 1990; Barnstable et al., 1983), amacrine (HPC-1 and VC1.1; Barnstable et al., 1985; Arimatsu et al., 1987), and Muller cells (Ret G1 and Ret G2; Barnstable, 1980) (Table 3 and Fig. 5); these are cell types normally generated postnatally. Few of the dividing cells expressed markers characteristic of rod photoreceptors (Ret P1; Akagawa and Barnstable, 1986). Several studies have also noted that rod photoreceptors do not develop in significant numbers in monolayer cultures of rat retina (Sparrow et al., 1990; Watanabe and Raff, 1990). Ret B1 is reported to label both bipolar and rod cells (Barnstable et al., 1983). Given the absence of rod cells in these cultures, the cells

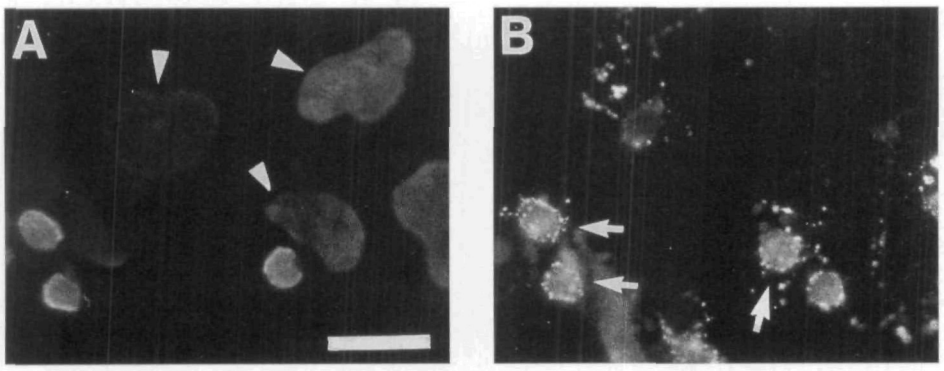

Fig. 5. Dividing cells later express a marker of bipolar cells. Three retinal progenitor cells which incorporated BrdU after 2 days in culture with bFGF (100 ng/ml; panel A, fluorescein) express a marker characteristic of bipolar cells after a total of 7 days in culture (panel B, texas red; antibody 115A10). Arrows in B indicate the cells with $BrdU^+$ nuclei shown in panel A. Cells with large nuclei (panel A, arrowheads) were not included in assessments of proliferation or differentiation (see Materials and methods). Bar, 20 μm.

labeled by Ret B1 are more likely to present bipolar cells than rod photoreceptors. Normally, approximately 9% of newborn retinal cells develop into bipolar cells (Turner and Cepko, 1987). The proportion of cells that expressed markers characteristic of bipolar cells was therefore higher than observed in the retina at equivalent ages (unpublished observation). A similar over-representation of cells expressing bipolar markers has been observed in other culture systems in which progenitor cells fail to develop into rods (Altshuler and Cepko, 1992). The small number of $GFAP^+$ cells is consistent with the expectation that using peripheral retina for these cultures selects against astrocytes and endothelial cells (Watanabe and Raff, 1988), which would be expected to divide in response to TGFα and FGFs (Raff et al., 1983; Burgess and Maciag, 1989). The low numbers of these non-retinal cells suggests that most of the dividing cells counted were retinal progenitor cells.

Responsiveness to FGFs and TGFα changes with age

To determine whether responsiveness to these mitogens changes during development, monolayer cultures prepared from E 15 - P 0 retinas were treated with different concentrations of FGFs or TGFα. In cultures of E15-E20 retinal cells, maximal proliferative responses were seen at very low concentrations of FGFs (Fig. 6). By contrast, higher concentrations of aFGF and bFGF were required in cultures of older retinal cells (Fig. 6). For example, the concentration required for half maximal response to bFGF increased approximately 40-fold between E15 and E21, and over 200-fold between E15 and P0. It is not clear whether the high concentrations of FGF required after birth for maximal responses are within a physiological range. The proportion of progenitor cells that divided in response to these saturating concentrations of FGFs also declined during development, particularly after E19 (Figs 4 and 6).

The dose-dependence of the mitotic response to TGFα also changed during development, but in an inverse, rather than parallel, manner (Fig. 7). Few progenitor cells in cultures of E15 retina divided in response to exogenous TGFα (Fig. 7A), though these cells responded to bFGF (Fig. 6). In cultures of E16.5 retinal cells, addition of TGFα stimulated proliferation, though a concentration of approximately 10 ng/ml was required to see a response (Fig. 7A). In contrast, 100 to 1000-fold less TGFα was needed to elicit maximal responses in cultures of E20-P0 retina (Fig. 7B). Although senstivity to TGFα increased during development, the overall proportion of progenitor cells that divided in response to TGFα declined (Figs 4 and 7). The postnatal retina thus appears to contain a subpopulation of progenitor cells that can divide in response to very low concentrations of TGFα.

Expression of TGFα in the retina

A recent study reported that TGFα mRNA could be detected in the developing rat retina between E15 and P7 (Anchan et al., 1991). TGFα is expressed in a variety

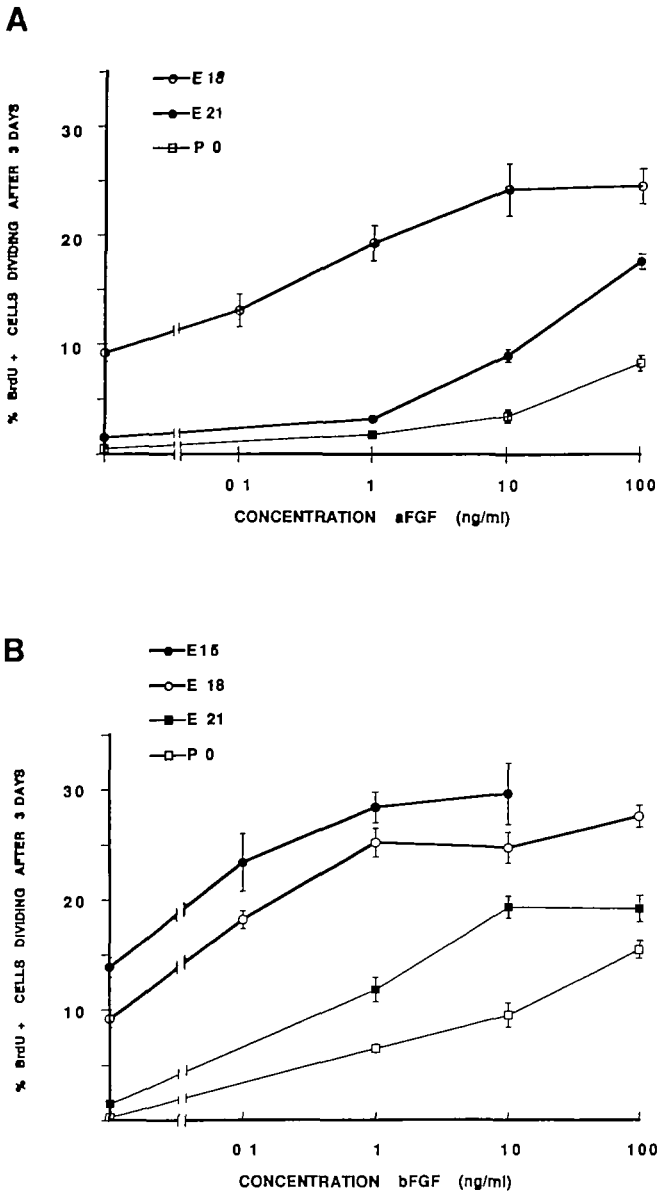

Fig. 6. Dose-dependence of mitogenic effects of FGFs. Monolayer cultures prepared at the indicated ages were exposed to different concentrations of aFGF (panel A) and bFGF (panel B). The concentrations required for half-maximal reponses to bFGF, for example, were: 0.01 ng/ml in E15 cultures, 0.03 ng/ml in E18 cultures, 0.4 ng/ml in E21 cultures, and 2.8 ng/ml in cultures of newborn retinal cells.

of tissues as a transmembrane precursor which can be cleaved through a regulated process to produce a diffusible $6\times10^3 M_r$ form (Pandiella and Massague, 1991). Both the membrane-associated and the diffusible forms of TGFα are capable of activating EGF receptors (Brachman et al., 1989; Wong et al., 1989) and stimulating proliferation (Anklesaria et al., 1990). In many of the systems studied, processing of pro-TGFα (membrane-associated) is incomplete, resulting in the accumulation of pro-TGFα on the plasma membrane (Anklesaria et al., 1990). If TGFα in the retina was

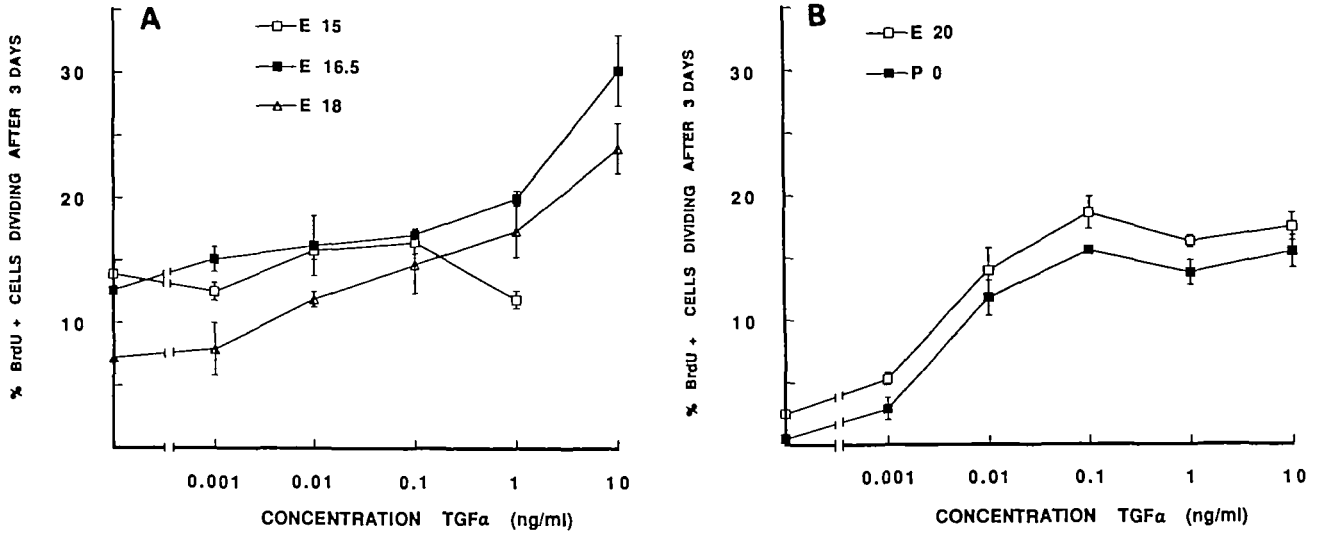

Fig. 7. Dose-dependence of mitogenic effects of TGFα. Monolayer cultures of E15-E18 retinal cells (panel A) and E20-newborn retinal cells (panel B) were exposed to different concentrations of TGFα

primarily of the membrane-associated form, it might be accessible to only those progenitor cells adjacent to the cells that expressed it. To determine the distribution of TGFα in the developing retina, sections of E16-P5 retina were stained with an antibody that specifically labels TGFα. As shown in Fig. 8, TGFα immunoreactive material was detectable throughout the retina and in the pigment epithelium from E16 through P5. This staining was blocked by an excess of TGFα (Fig. 8J), but not by EGF (Fig. 8I). This suggests that TGFα would be accessible to most retinal progenitor cells during the period of retinal proliferation, even if it was predominantly membrane-associated rather than diffusible.

Discussion

We have found that several growth factors normally produced by the retina and surrounding ocular tissue stimulate the proliferation of retinal cells in vitro. Moreover, the responsiveness of retinal cells to these growth factors changes during development. Many, if not all, of the dividing cells appear to be retinal progenitor cells, as they subsequently express markers characteristic of differentiated retinal cells. These results suggest that TGFα and FGF, or related factors, may contribute to the control of proliferation in vivo. These findings confirm and extend previous work which suggested that retinal progenitor cells change during development with respect to proliferation (Watanabe and Raff, 1990; Taylor and Reh, 1990). These observations also raise questions about the mechanisms that underlie these changes, and the relationship of changes in mitogen responsiveness to the development of specific types of retinal cells.

Are TGFα and FGF endogenous mitogens?

The retina has been reported to produce several members of the FGF family of growth factors, including aFGF, bFGF, and int-2 (Noji et al., 1990; Wilkinson et al., 1989). TGFα, a member of the EGF family of growth factors, is also present in the developing rodent retina (Anchan et al., 1991, and this report). Growth factors belonging to both families might be expected to act locally, rather than diffusing over large distances: factors related to FGF share the property of binding to heparan-sulfate proteoglycans of the extracellular matrix and cell surfaces (reviewed by Burgess and Maciag, 1989), and TGFα (or EGF) may be expressed predominantly as a membrane-bound form (Pandiella and Massague, 1991). Consistent with these properties, we did not find evidence that the endogenous mitogenic signals produced by retinal explants behaved as freely diffusible factors. The presence of members of these growth factor families in the retina in vivo, together with their mitogenic effect on retinal progenitor cells in vitro, strongly suggests that TGFα and FGF, or related factors, play a role in the control of proliferation in the retina in vivo. Though it remains to be determined whether these factors act directly on retinal progenitor cells, it has been reported that retinal progenitor cells express mRNA for FGF receptors, suggesting that a direct effect of FGF is at least possible (Wanaka et al., 1991; Heuer et al., 1990). EGF receptor has also been observed in the rat retina, though its cellular localization was not determined (Anchan et al., 1991). In order to confirm the role of FGFs and TGFα in the proliferation of retinal progenitor cells in vivo, it will be necessary to determine whether blocking their action in vivo reduces proliferation. Recent reports have demonstrated that introduction of mutated forms of FGF and EGF receptors can act in a dominant negative manner to inhibit function in vitro and in vivo (Amaya et al.,

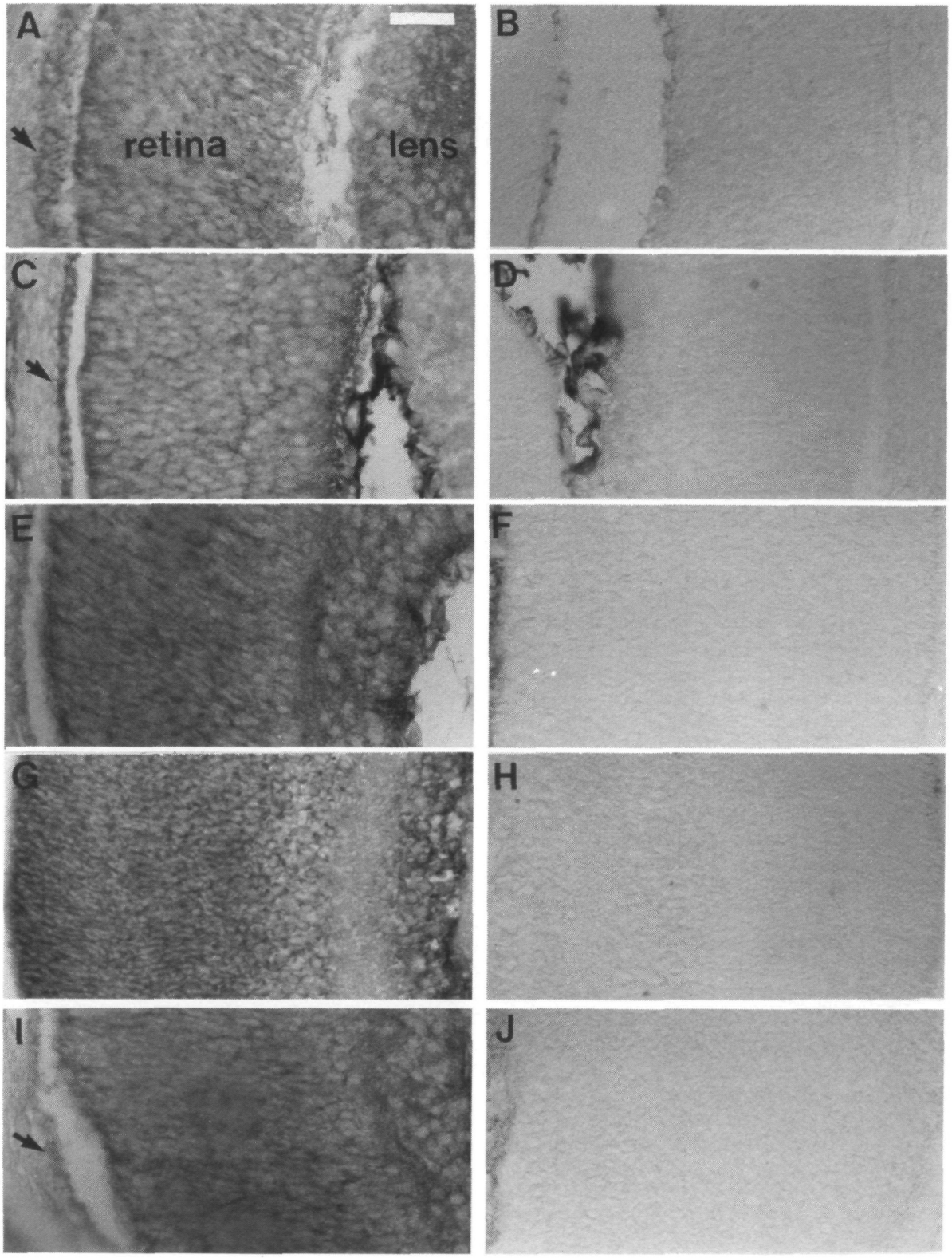

Fig. 8. Sections of eyes from E16.5-P5 stained with an antibody to TGFα. A,C,E and G show sections stained with TGFα antibody, B,D,F and H show sections stained with an irrelevant monoclonal antibody (BrdU): (A and B) E16.5, (C and D) E18, (E and F) P1, (G and H) P5. The TGFα antibody labels retina, pigment epithelium (arrows), and lens. I and J show sections of P1 eye stained with TGFα antibody after preincubation with TGFα (J), or with EGF (I). Note that staining is blocked by preincubation with TGFα but not EGF. Labeling of the vitreous is non-specific. Bar, 50 μm.

1991; Kashles et al., 1991). Introduction of such receptor constructs may be a way of assessing the role of these and related factors in vivo.

Even if TGFα and FGF-related factors are involved in the stimulation of proliferation in vivo, it is not clear that they are the only mitogenic signals involved, or that the retina is the only source of mitogens that act on retinal progenitor cells. When proliferation in explants was compared to proliferation in vivo at equivalent ages, proliferation in vitro was always lower, by a factor of approximately two. This discrepancy could indicate that additional mitogenic signals remain to be identified, particularly at the early ages that were not affected by exogenous TGFα or FGFs in explant cultures. Such signal(s) could be produced outside the retina, for example, by the pigment epithelium (PE). The PE apposes the ventricular surface of the retina in vivo, but was removed in explant preparations. PE cells have been reported to produce a variety of growth factors (Millan et al., 1991; Schweigerer et al., 1987); the PE could therefore provide additional mitogenic signals, or could stimulate retinal production of such signals. Alternatively, the extracellular matrix that lies between the PE and the retina may enhance production of mitogenic signals, or increase the responsiveness of retinal progenitor cells to such signals; similar roles for extracellular matrix have been suggested by studies in retina (Reh and Radke, 1988) as well as many other systems (reviewed by Nathan and Sporn, 1991). This extracellular matrix material may also be absent or limiting in explant preparations. Co-culture of retinal explants with PE, or PE matrix, would help to address these possibilities, and perhaps lead to the identification of additional mitogenic signals.

Progenitor cells change during development

The results presented in this study demonstrate that the responsiveness of retinal progenitor cells to the mitogenic effects of both TGFα and FGFs in vitro changes during development, and the change is complementary rather than parallel. Previous studies have also suggested that retinal progenitor cells change during development (Taylor and Reh, 1990; Watanabe and Raff, 1990). For example, Watanabe and Raff reported differences in the expression of a rod phenotype in E15 vs P1 retinal cells in a shared culture environment. This study also demonstrated a difference in the proliferative response of progenitor cells from E 15 and P1 retinas to the same environment in vitro, though the signals involved were not identified. The basis of these changes, intrinsic versus environmental, has not yet been identified, nor has the level at which the changes occur. Moreover, it is possible that the apparent changes in rod development and proliferation are linked.

Responsiveness to FGF has been shown to be regulated by a combination of heparan sulfate proteoglycans and tyrosine kinase receptors (reveiwed by Klagsbrun and Baird, 1991). In other systems, loss of responsiveness to FGF has been shown to be associated with loss of FGF receptors (Olwin and Haushka, 1988; Iwamoto et al., 1991) or deficiencies in heparin-like molecules (Yayon et al., 1991; Bernard et al., 1991; Rapraeger et al. 1991). Alternatively, since several types of FGF receptor have been identified, changes in responsiveness to FGF could reflect a change in the type of FGF receptor that is expressed (Johnson et al., 1990; Keegan et al., 1991; Partanen et al., 1991; Lai and Lemke, 1991). The decline in responsiveness to FGF appears to precede withdrawal from the cell cycle, as cells can be pushed to continue to divide by raising the concentration of FGF. This suggests that the decline in responsiveness precedes loss of proliferative capacity, and may therefore play a causal role in limiting proliferation. A mechanism of this kind underlying the control of proliferation would easily accommodate a situation in which proliferation and differentiation occur in a common microenvironment.

The onset of a mitotic response to exogenous TGFα seen after E15, and the subsequent reduction in the concentration of TGFα needed to stimulate maximal proliferative responses could be due to several kinds of mechanisms as well. For example, these changes could reflect the onset of expression of EGF receptors (which mediate responses to TGFα) by retinal progenitor cells, and the change in dose-dependence of proliferation could then be due to increased receptor density. Alternatively, the changes in responsiveness to TGFα could be due to changes in signal transduction, or a switch in the type of receptor expressed; most responses are thought to be mediated by the EGF receptor, but a novel tyrosine kinase related to the EGF receptor has recently been described (Lai and Lemke, 1991). It has not yet been determined, however, whether this EGF receptor-like molecule can be activated by TGFα.

Why do progenitor cells stop dividing?

Several kinds of mechanisms could contribute to the restriction of proliferation in the retina. Recent transplant studies in the chick retina suggest that environmental signals that regulate proliferation may change during development, as the environment found in early embryonic retina supports more extensive proliferation than does an older retinal environment (D. Fekete, et al., 1990). It is not likely, however, that retinal progenitor cells stop dividing only because concentrations of mitogens become limiting at late embryonic stages; for example addition of excess TGFα and FGFs in vitro does not prevent progenitor cells from dropping out of division. While it is possible that the decline in proliferation reflects the fall of a mitogen that has not yet been identified, the study by Watanabe and Raff (1990) also suggested that environmental signals alone were not the limiting factors in the control of retinal progenitor cell division. We observed that the mitotic responsiveness of progenitor cells to FGF declined between E18 and the time of birth, as did the size of the population of progenitor cells that responded to either TGFα or FGF. These changes could be due to either an intrinsic developmental program or to extracellular signals.

While we have focused on mitogenic signals, inhibi-

tory environmental signals could also contribute to the control of proliferation in the retina, perhaps by regulating responsiveness to mitogenic signals. For example, it has been observed that TGFβ_1 inhibits proliferation in cultures of human retinal cells (Kimchi, et al., 1988). Preliminary results (Lillien and Cepko, unpublished) indicate that the rodent retina produces inhibitory signals related to TGFβ, as we have observed that a neutralizing antiserum to TGFβ enhances proliferation in cultures of rat retinal cells, an effect which can be blocked by excess TGFβ_2. TGFβ has been shown to inhibit proliferation in other systems, including responses mediated by FGF and EGF, by modulating mitogen signaling pathways (Takehara et al., 1987; Muller et al., 1987). Though TGFβ could contribute to the restriction of proliferation in the retina, it would have to do so in a way that permitted neighboring cells to continue dividing. As with the mitogenic signals, responsiveness to TGFβ, or other inhibitory signals, may be developmentally regulated.

Implications for cell type determination

Retinal progenitor cells have been shown to be multipotent (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). It has been proposed that cell identity may be induced by environmental signals, and that this may occur at or after the time of a cell's last division (Turner and Cepko, 1988; Turner et al., 1990). A correlation between choice of cell type and the time of withdrawal from the cell cycle has been shown by analysis of birthdates: cells that stop dividing before E16, for example, tend to develop into cones, ganglion, and horizontal cells, while cells that stop dividing after E16 tend to develop instead into amacrine, rod, bipolar and Muller cells (M. LaVail, D. Yasumura, and D.H. Rapaport, personal communication). By determining when progenitor cells divide, the mechanisms used to control proliferation in the retina may therefore also directly or indirectly influence the specification of cell type. The onset of the mitotic response to TGFα occurs at approximately the same time that cells switch from early to late cell fates. It would be interesting to determine, for example, whether acquisition of mitotic responsiveness to TGFα is associated with this change in cell type choice.

While lineage studies suggest that some cell types are determined around the time of a cell's last division, it is not clear whether this is true for all cell types, or whether cells must become postmitotic, or decide that they have entered a terminal cell cycle, before they can respond to environmental signals that determine cell type. Studies in the chick retina, for example, indicate that immature ganglion cells continue to divide after expressing a differentiation marker such as neurofilament (Guillemot and Cepko, 1992). Preliminary experiments (Lillien and Cepko, unpublished data) suggest that both FGF and TGFα affect the determination of cell type, in that they inhibit the differentiation of postnatal rodent progenitor cells into rods. They do not inhibit differentiation into amacrine or bipolar cells, however, indicating that they do not inhibit differentiation generally. It is not yet clear whether these effects are due to the mitogenic action of these growth factors, or if they inhibit rod development by acting on postmitotic cells. If these effects are due to the mitogenic action of these factors, these preliminary studies suggest that some inducing signals may act only on postmitotic cells, while others can act on cells before they become postmitotic. This raises the possibility that signals that determine cell type may themselves inhibit proliferation.

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