Short communication

Retinal and olfactory bulb precursor cells show distinct responses to FGF-2 and laminin

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Abstract

We analyzed whether the embryonic (E12.5–E14.5) mouse retina possesses genuine neural stem cells and how they respond to defined growth factors and extracellular matrix molecules. Whereas most combinations produced no or limited cell survival and proliferation in culture, FGF-2 plus heparin and laminin stimulated proliferation and the formation of aggregates composed, after two days, of 95.2% nestin-positive cells. However, cells in these aggregates could only be passaged poorly, lost nestin expression and proliferative capacity, and differentiated into neurons. Under the same conditions, olfactory bulb precursor cells divided efficiently and could be expanded. These data suggest that, in addition to FGF-2 and laminin, embryonic retinal neuroepithelial cells need additional extrinsic and/or intrinsic regulators to maintain cell proliferation and self-renewal.

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1. Introduction

At early stages of embryonic development, neuroepithelial precursor cells of most vertebrate nervous system regions have the potential to differentiate into neurons, astrocytes, and oligodendrocytes and the ability to self-renew, indicating that they are neural stem cells (NSC) (Temple, 2001; Vicario-Abejón et al., 2003). NSC have also been isolated from the mammalian adult central nervous system (CNS), including the adult retina (Tropepe et al., 2000; Temple, 2001). Radial glia, such as retinal Müller glia, can also function as neuronal precursor cells (Malatesta et al., 2000; Fischer and Reh, 2001). Early studies reported multipotent precursor cells in the developing neuroretina (Turner and Cepko, 1987; Wetts and Fraser, 1988), although the ability of these precursors to self-renew

has not been demonstrated (Akagi et al., 2003; Bhattacharya et al., 2003; James et al., 2003; Ahmad et al., 2004). Most NSC proliferate in culture in response to FGF-2 and EGF in combination with IGF-I and (pro)insulin (Vicario-Abejón et al., 2003). In addition, extracellular matrix (ECM) molecules are also involved in the regulation of precursor cell proliferation in the CNS (Reh and Radke, 1988; Blaess et al., 2004).

Here we studied whether the embryonic mouse retina possesses genuine NSC, and analyzed their responses to defined extracellular factors and ECM molecules. In parallel, we analyzed the responses of well-characterized olfactory bulb stem cells (OBSC; Vicario-Abejón et al., 2003; Yusta-Boyo et al., 2004) to the same factors. In contrast to cells isolated from the olfactory bulb (OB), retinal precursors do not form neurospheres in response to FGF-2. Nonetheless, retinal cells respond to a combination of FGF-2, heparin and laminin by proliferating and forming cell aggregates. These aggregates, however, tend to differentiate in short-term culture and can be expanded poorly. Our results suggest that specific and distinct responses of embryonic retinal neuroepithelial precursors

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Nomenclature

BrdU	5'-bromo-2-deoxyuridine
CNTF	ciliary neurotrophic factor
E	embryonic day of development
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
IGF	insulin-like growth factor
NCS	neural stem cells
NT	neurotrophin
OB	olfactory bulb
OBSC	olfactory bulb stem cells
PFA	paraformaldehyde
TGF	transforming growth factor

to mitogens and ECM molecules are linked to their potential ability for self-renewal in culture.

2. Materials and methods

2.1. Cell culture

Reagents for tissue culture were purchased from Gibco-Life Technologies (Carlsbad, CA, USA), Sigma (St. Louis, MO, USA) and Worthington (Freehold, NJ, USA). FGF-2, IGF-I, EGF, CNTF, and TGF- α were purchased from PeproTech (Rocky Hill, NJ, USA), NT-3 was from R&D Systems (Minneapolis, MN, USA); insulin was a kind gift of Eli Lilly (Indianapolis, IN, USA). Heparin and Matrigel were purchased from Serva (Heidelberg, Germany).

Cell cultures were prepared from CD1 mouse embryonic retina on gestational days 12.5, 13.5, and 14.5 (E12.5–E14.5) and from E14.5 olfactory bulb. Animals were cared for in accordance with European Commission guidelines. Whole retinas were dissected out of the eye by separating them from the optical nerve; the pigmented epithelium and the lens were then removed. Cells were obtained by mechanical dissociation of pooled neuroretinas, followed by mild trypsinization. E12.5 whole retinas were first treated with hyaluronidase. After inhibition of enzyme activity with 10% heat-inactivated FBS, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12/insulin, apotransferrin, putrescine, progesterone, sodium selenite (N2 supplements), plated on tissue culture dishes and incubated (37 °C, 5% CO₂). While establishing the culture system, initial cell densities in the range of 35,000–80,000 cells/cm² were employed without noticeable differences. Thus, 60,000 cells/cm² were chosen for further experiments. Defined growth factors and ECM molecules were added daily or every other day.

E14.5 OB cell cultures were prepared by mechanical dissociation and plated on uncoated tissue culture dishes at a density of 35,000 cells/cm² as described (Vicario-Abejon et al., 2003; Yusta-Boyo et al., 2004). FGF-2 (50 ng/ml), heparin (2 μ g/ml) and laminin (2 μ g/ml) were added to expand the proliferative precursor cell population. Under these conditions, OB cells grew as monolayers and were passaged every 3–5 days by mechanical procedures and plated at 5000 cells/cm² for passage.

For cell proliferation assays, cells were pulsed with 5 μ M 5'-bromo-2-deoxyuridine (BrdU; Boehringer-Mannheim) for 20–22 h prior to fixation.

2.2. Immunostaining of cultured cells and sections

Cultured cells were fixed in 4% paraformaldehyde (PFA)/0.1 M phosphate buffer, pH 7.4, for 25 min. Cultures incubated with BrdU were fixed in 4% PFA/0.1 M borate (Na₂B₄O₇) buffer, pH 9.5, for 25 min, treated with 2 N HCl for 10 min, and neutralized in 0.1 M Na₂B₄O₇ for 10 min. After treatment with 0.1% Triton X-100/10% normal serum/PBS, cells were incubated (4 °C,

overnight) with primary antibodies against nestin (rabbit polyclonal, 1:1000; a kind gift of R.D. McKay, NIH, Bethesda, MD, USA), BrdU (mouse monoclonal, 1:1000–1:2000), and β -III-tubulin (TuJ1, rabbit polyclonal, 1:2000–1:4000; Babco, Richmond, CA, USA). Cells were then incubated with the appropriate Alexa fluor 488- or Texas red-conjugated secondary antibodies (1:100; Molecular Probes, Eugene, OR, USA). Controls were performed to confirm antibody specificity. The anti-BrdU monoclonal antibody (G3G4) developed by S.J. Kaufman was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA.

For histology, E12.5–E13.5 heads were fixed overnight in 4% PFA in PBS immersed in 30% sucrose for 24–48 h, then frozen at –70 °C in dry ice. Air-dried cryostat coronal sections (14 μ m) were incubated overnight at 4 °C or at room temperature with the polyclonal primary antibodies anti-nestin, TuJ1 (1:1500), and anti-phosphohistone H3 (rabbit polyclonal, 1:1000 from Upstate, Lake Placid, NY, USA).

2.3. Cell counts and statistical analysis

To determine the number of cells expressing a specific antigen, a total of 10 random fields per chamber were counted using $\times 20$ or $\times 40$ objectives under fluorescence filters. Results are expressed as the number of cells stained for that antigen in 10 fields. Total cell number (stained plus unstained) was counted to calculate the proportion of a specific cell type in the culture. Results are expressed as the mean $\pm$ SEM of data from four cultures of two experiments.

3. Results

3.1. Cell marker expression in the developing retina in vivo

The expression patterns of nestin (a neuroepithelial cell marker), β -III-tubulin (TuJ1, a neuronal marker), and phosphohistone H3 (a mitosis marker) were studied on E12.5–E13.5 sections (for references on cell markers, see Ref. Vicario-Abejon et al., 2003). TuJ1-positive cells were located near the optic nerve in E12.5 retinas (Fig. 1A), whereas these cells were distributed throughout E13.5 retinas, with the exception of the edges of the peripheral retina (Fig. 1C, E). Nestin expression was abundant in the E13.5 retina (Fig. 1F) and its distribution pattern partially overlapped that of TuJ1. Phosphohistone H3-positive cells (Fig. 1B, D) were distributed throughout the neuroepithelial zone and were more abundant at E12.5 than at E13.5.

3.2. Effects of defined molecules on retinal and olfactory bulb precursor cells

Since nestin-positive cells were abundant in the developing retina (Fig. 1F), cells were isolated and plated in culture to attempt expanding the proliferative precursor cells using growth factor and ECM molecule treatment (Anchan et al., 1991; Klagsbrun and Baird, 1991; Lillien and Cepko, 1992; Hernandez-Sanchez et al., 1995; Das et al., 2000; Vicario-Abejon et al., 2003). Retinal cell survival was poor under most conditions (Table 1). Of the factors tested, only FGF-2 (50 ng/ml) plus heparin (2 μ g/ml) (in polyornithine-coated dishes) promoted formation of small aggregates, although with low efficiency. Since cell responses to growth factors

Fig. 1. Cell marker expression in the developing retina *in vivo*. Coronal sections from E12.5–E13.5 mouse retinas were immunostained with antibodies specific for β -III-tubulin (TuJ-1; A, C, E), phospho-histone H3 (B, D), and nestin (F). Panel E shows a high magnification image of the area indicated in C with an arrowhead. The asterisk indicates the site in the ganglion cell layer at which the optic nerve leaves the retina. L, lens. Scale bar (shown in F), A–D, 50 μ m; E–F, 20 μ m.

can be modulated by ECM molecules (Colognato et al., 2002), Matrigel (0.4–1%), laminin (1–2 μ g/ml) and fibronectin (1–2 μ g/ml) were added to the cultures in the absence of polyornithine. The combination of FGF-2, heparin and laminin produced a significant increase in the number and size of cell aggregates after three to four days in culture (Table 1; Fig. 2), whereas fibronectin had no significant effects.

Matrigel, which contains high proportion of laminin (Xu et al., 2001), also stimulated aggregate formation in combination with FGF-2, whereas Matrigel alone had no effect. Cell

density effect in the range of 35,000–80,000 cells/cm² was minor, with slightly better aspect of the cultures at higher densities. After two days of treatment with FGF-2 plus heparin plus laminin, the proportions of TuJ1-, BrdU-, and nestin-positive cells were 44.9%, 45.2%, and 95.2%, respectively (Fig. 3A–F, I). Passage of these aggregates was inefficient, however, and the cells could not be expanded. In addition, the proportions of nestin- and BrdU-positive cells decreased over time, whereas the proportion of TuJ1-positive cells increased (Fig. 3I), even at high cell density, a fact that appears

Table 1
Effects of defined molecules on retinal precursor cell growth

Treatment	Growth
FGF-2 20–50 ng/ml	–
FGF-2 20 ng/ml + EGF 20 ng/ml	–
FGF-2 50 ng/ml + heparin 2 µg/ml + polyornithine	+/-
FGF-2 50 ng/ml + heparin 2 µg/ml + Matrigel 0.4–1%	+
FGF-2 50 ng/ml + heparin 2 µg/ml + fibronectin 1–2 µg/ml	–
FGF-2 50 ng/ml + heparin 2 µg/ml + laminin 1 µg/ml	++
FGF-2 50 ng/ml + heparin 2 µg/ml + laminin 2 µg/ml	+++

Mouse E13.5 retinal precursor cells were cultured with the indicated growth factors and ECM molecules, and 10 µg/ml insulin (except when IGF-I was tested). Cell survival and formation of cell aggregates in the cultures were evaluated four days after plating (see Fig. 2). As shown, the combination of 50 ng/ml FGF-2, 2 µg/ml heparin and 2 µg/ml laminin produced the best results and was chosen for standard growth conditions for retinal cells. Other factors and combinations tested that did not promote growth were EGF, IGF-I, NT-3, TGF- α , CNTF, Matrigel, FGF-2 + NT-3, FGF-2 + TGF- α , IGF-I + Matrigel, FGF-2 + polyornithine.

to minimize differentiation of human retinal precursor cells (Ezeonu et al., 2003). These results suggest that the retinal nestin- and BrdU-positive cells do not possess self-renewal capacity under these culture conditions. They also indicate that laminin potentiates the survival and proliferative responses of retinal cells to FGF-2.

Since laminin can also promote neuroepithelial cell differentiation (Wu et al., 2002), and this could be a reason for the increase in the neuronal marker expression in the retinal cultures, OB precursor cells were plated under the same conditions. After two days, the proportions of TuJ1-, BrdU-, and nestin-positive cells were 83.2%, 15.7%, and 93.3%, respectively (Fig. 3I). In contrast to retinal cells (Fig. 3A–F), OB precursors grew as monolayers (Fig. 3G, H) and could be passaged efficiently; by day eleven the proportion of BrdU- and nestin-positive cells increased to 98% and 99.5%, respectively, whereas the proportion of TuJ1-positive cells was 0.06%.

4. Discussion

Neuroepithelial precursor cells showing stem cell features have been isolated from the embryonic retinal pigmented epithelium (Coles et al., 2006) and the adult mammalian ciliary margin (Trobepe et al., 2000; Ahmad et al., 2004). In contrast, although multipotent precursors were reported in the developing retina (Turner and Cepko, 1987; Wetts and Fraser, 1988), no precursor cell with self-renewal capacity has been isolated from the embryonic neuroretina (Akagi et al., 2003; Bhattacharya et al., 2003; James et al., 2003; Ahmad et al., 2004). We show that early embryonic mouse retinal precursor cells do not survive significantly or proliferate in culture, in the presence of defined growth factors or growth factor-combinations (FGF-2, EGF, IGF-I, NT-3, CNTF and TGF- α) in medium containing a high insulin concentration. As cell dissociation may produce loss of critical interactions between growth factors and their receptors and/or between cell membranes and the extracellular matrix, necessary for cell survival

Fig. 2. Growth of early embryonic retinal precursor cells in culture. E13.5 retinal cell suspensions were plated on uncoated tissue culture dishes in the presence of 50 ng/ml FGF-2, 2 µg/ml heparin and 2 µg/ml laminin. A, One day after plating, many cells are seen in the cultures. Only a small percentage of these cells survived and formed small and large aggregates at days 2 (B) and 4 (C). Only minor differences were observed when the cells were plated at initial densities in the range of 35,000–80,000 cells/cm². E14.5 cell growth was similar to that of E13.5 cells; E12.5 cells grew poorly under these culture conditions. Scale bar (shown in C), 40 µm.

and proliferation, we studied the effects of ECM molecules in these processes.

The very weak effect of FGF-2 plus heparin on the formation of retinal cell aggregates is strongly and specifically

Fig. 3. Cell marker expression in retinal and olfactory bulb precursor cells in culture. Retinal (A–F) and olfactory bulb (G, H) cell suspensions were plated on uncoated tissue culture dishes in the presence of 50 ng/ml FGF-2, 2 μ g/ml heparin and 2 μ g/ml laminin. BrdU (5 μ M) was added to the cultures to label proliferating cells. Cells were fixed on different days and immunostained with antibodies against β -III-tubulin (TuJ1; A, B), BrdU (C, D, G), and nestin (E, F, H). The same fields are shown in A, C and E, in B, D and F, and in G and H, respectively. I, the graph shows a time-course of the proportions of TuJ1-, BrdU-, and nestin-positive cells in retinal (left) and olfactory bulb (OB, right) cultures. Note that whereas the percentage of BrdU-positive cells in OB cultures increased with time, retinal cells differentiated and lost nestin expression and proliferative capacity. Results are expressed as the mean $\pm$ SEM of data from four cultures of two experiments. Scale bar (shown in H), A, C, E, G, 50 μ m; B, D, F, H, 20 μ m.

potentiated by laminin, but this molecule (or Matrigel) alone has no effect. This indicates that laminin is necessary for FGF-2 promotion of retinal precursor cell survival and proliferation as well as aggregation. This cooperative effect between the two proteins, however, does not prevent the loss of nestin expression and induction of neuronal differentiation with time in culture. Although laminin may promote cell differentiation (Wu et al., 2002), which could explain the increase in the number of TuJ1-positive cells in the retinal cultures, OBSC proliferate rapidly in the presence of laminin. These data suggest that, in addition to FGF-2 and laminin, embryonic retinal neuroepithelial cells may need additional signals, including the activation of signaling molecules and transcription factors involved in the stimulation of cell proliferation and self-renewal, which may occur later in development.

In support of this, late embryonic (E18.5) and early postnatal (P1) retinal precursor cells proliferate (but do not self-renew) in the presence of EGF (James et al., 2003; Ahmad et al., 2004; Klassen et al., 2004), and adult mouse retinal cells proliferate and self-renew in response to FGF-2, heparin and EGF (Trobepe et al., 2000). Activation of the Delta-Notch pathway may also be important for maintenance of neuroepithelial cells in a precursor state. Rat retinal stem cells isolated from E14 and E18 embryos as well as from the adult ciliary margin express Notch, whereas expression of its ligand Delta increases from background levels at E14 to significant levels in the adult. In addition, signaling through the Wnt-Frizzled pathway and activation of the c-kit receptor tyrosine kinase by its ligand stem cell factor, promotes neurosphere formation by adult ciliary margin stem cells (Ahmad et al., 2004).

Neuroepithelial precursor cells from many CNS regions tend to differentiate in culture (Reh and Kljavin, 1989; Vicario-Abejon et al., 1995). To our knowledge, with the exception of embryonic retinal cells, the precursors can be stimulated to proliferate and self-renew in response to FGF-2, EGF and members of the insulin family of growth factors (Vicario-Abejon et al., 2003). In contrast, the data presented here and results from other groups (Ahmad et al., 2004) suggest that proliferation (and possibly the ability to self-renew) of retinal precursor cells is finely regulated during development and adult life, by activation of a number of signaling components, including activation of the FGF-2 receptor tyrosine kinase signaling pathway by laminin. Understanding the differences between neuroepithelial cells from different regions might facilitate their manipulation *in vitro*, and eventually shed light on their behavior *in vivo*, with the long-term aim of using them for treating CNS disorders.

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