

Embryonic Cerebrospinal Fluid Regulates Neuroepithelial Survival, Proliferation, and Neurogenesis in Chick Embryos

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ABSTRACT

Early in development, the behavior of neuroepithelial cells is controlled by several factors, which act in a developmentally regulated manner. Diffusible factors are secreted locally by the neuroepithelium itself, although other nearby structures may also be involved. Evidence suggests a physiological role for the cerebrospinal fluid in the development of the brain. Here, using organotypic cultures of chick embryo neuroepithelial explants from the mesencephalon, we show that the neuroepithelium *in vitro* is not able to self-induce cell survival, replication, and neurogenesis. We also show that the embryonic cerebrospinal fluid (E-CSF) promotes neuroepithelial stem cell survival and induces proliferation and neurogenesis in mesencephalic explants. These data strongly suggest that E-CSF is involved in the regulation of neuroepithelial cells behavior, supporting the hypothesis that this fluid plays a key role during the early development of the central nervous system. © 2005 Wiley-Liss, Inc.

Key words: brain development; neural tube; neural differentiation; neuroepithelium tissue culture; mesencephalon; neural stem cell

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube. The development of a complex structure such as the central nervous system (CNS) from a relatively simple primordium involves the simultaneous and interdependent action of various developmental mechanisms, including morphogenetic mechanisms, the establishment of positional identities, and the complex process of histogenesis. The individual regulation and coordination among these processes are only partially known.

During early stages of development, the brain wall is formed by a pseudostratified neuroepithelium composed of rapidly proliferating precursors that maintain connections to both the ventricular and the pial surface (Panchision and McKay, 2002). This neuroepithelial

wall also encloses the brain cavity, which is filled with the E-CSF.

It has been demonstrated that some of the molecular mechanisms involved in the control of the cellular behavior and patterning of the CNS come from the neuroepithe-

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lium itself. Thus, many studies on the development of the CNS have focused exclusively on neuroepithelial tissue. The pattern of gene expression in brain vesicles, whose spatial and temporal dynamics are developmentally regulated, and the action of many of these genes on the patterning of CNS structures have been established. For example, the differential expression of several Hox genes in the hindbrain is responsible for the particular rhombomere identity (Duboule, 1994; Gaufo et al., 2003) and the expression of other transcription factors, e.g., *emx*, *otx*, and *en*, among others, accounts for the patterning of the cephalic mid- and forebrain (Bally-Cuif and Bonicelli, 1997).

On the other hand, several groups of cells that act as organizing centers have also been identified within the neuroepithelium, namely, the floor plate, the mesencephalic-rhombencephalic isthmus, and the most anterior part of the telencephalic vesicles, and in adjacent structures such as the notochord (Placzek et al., 1991, 1993; Yamada et al., 1991; Roelink et al., 1994; Crossley and Martin, 1995; Bueno et al., 1996; Crossley et al., 1996). These organizing centers produce diffusible molecules, e.g., growth factors and morphogens, that are recognized by the receptors of cells within the organizing center or at a certain distance from it. These secreted molecules are involved in the establishment of positional identities and in the patterning of structures during CNS growth and development. Many of the molecules that exert their actions from these organizing centers have been identified, and they include several members of the *fgf* family and *shh*, among others (Bueno et al., 1996; Shamim et al., 1999; Vaccarino et al., 1999a, 1999b; Toresson et al., 2000; Garda et al., 2001; Panchision and McKay, 2002).

During histogenesis, neuroepithelial cells show a highly dynamic cellular behavior. Initially, the neuroepithelial wall is mainly built up of neural precursors, which readily fulfill two criteria for stem cells: capacity for self-renewal and multipotency. The brain neuroepithelium quickly evolves from an initial and intense proliferation phase, during which neuroepithelial stem cells are driven to increase the number of progenitor cells, to a differentiation phase, which drives neuroepithelial stem cells first to neurogenesis and later to gliogenesis (Panchision and McKay, 2002).

Except for the known effect of the notochord on the floor plate of the neural tube through the secretion of *shh* (Echelard et al., 1993; Martí et al., 1995), several reports indicate that the neuroepithelium shows autonomous behavior, which is exerted by the autocrine and/or paracrine secretion of diffusible molecules (Vaccarino et al., 1999b). Certain growth factors have been implicated in the control of neuroepithelial stem cell proliferation, neurogenesis, and gliogenesis, e.g., FGF2 and EGF, which may be involved in the control of replication and neurogenesis at early developmental stages (Gensburger et al., 1987; Murphy et al., 1990; Kilpatrick and Bartlett, 1993; Tropepe et al., 1999; Vaccarino et al., 1999a, 1999b; Panchision and McKay, 2002; Rajan et al., 2003).

However, the architecture of the brain primordium reveals another component, the cavity of brain vesicles, which is filled by E-CSF, a protein-rich fluid. The role of this fluid has not been as deeply analyzed as that of the rest of the components involved in brain development at early developmental stages (i.e., the neuroectoderm itself

and the organizing centers), although it may influence neuroepithelial stem cells. In this way, several studies have demonstrated that, during development, the most prevalent components of the E-CSF are proteins. Moreover, the fact that the protein fraction of the CSF is more complex in embryos than in adults, and that it is detected at higher concentrations, has led some authors to suggest that the E-CSF is involved in the regulation of neuroepithelial cell behavior (Dziegielewska et al., 1980b, 2000; Ojeda and Piedra, 2000; Miyan et al., 2003; Gato et al., 2004). In addition, it has been shown that E-CSF plays a key role in the expansion of the embryonic brain primordium at the earliest stages of development (Desmond and Jacobson, 1977; Desmond, 1985; Gato et al., 1993; Alonso et al., 1998, 1999; Desmond and Levitan, 2002). CSF influences brain development and cortical histogenesis at fetal and early posthatching stages (Miyan et al., 2003). Moreover, an altered CSF in some congenital malformations has been associated with variations in cortical histogenesis (Mashayekhi et al., 2002; Owen-Lynch et al., 2003).

The aim of this study is to demonstrate that the diffusible molecules of the E-CSF have a direct role in the survival, proliferation, and differentiation of the neuroepithelial cells. To test this hypothesis, we developed an organotypic tissue culture technique for explants of the mesencephalic neuroepithelium that allowed us to analyze the cellular behavior of these explants in a simplified system. This culture system maintains the neuroepithelium architecture with its intrinsic cell-cell interactions and eliminates the influences exerted by other embryonic structures. First, to assess the validity of this technique, we compared the cellular behavior of these explants cultured with FCS-supplemented medium and that of embryos maintained *in vivo*. Second, to test whether the cellular behavior of neuroepithelial cells is autonomous or depends, at least partially, on stimuli other than those of the neuroepithelium itself, we compared the cellular behavior of these explants cultured *in vitro* with a chemically defined serum-free medium and that of explants cultured with FCS-supplemented medium and the embryos maintained *in vivo*. Third, to test whether the E-CSF conditions the cellular behavior of neuroepithelial cells, we compared the data obtained in the above experiments with data obtained after culturing mesencephalic neuroepithelial explants with E-CSF-supplemented medium.

Here, we demonstrate that the early mesencephalic neuroepithelium cannot self-stimulate its basic cellular behavior *in vitro*, and that normal behavior is, to a great extent, promoted by extraneural signals that seem to be present in the E-CSF as diffusible factors.

MATERIALS AND METHODS

Obtaining Embryonic Cerebrospinal Fluid

Fertile chicken eggs were incubated at 38°C in a humidified atmosphere to obtain chick embryos at developmental stage HH25 (Hamburger and Hamilton, 1951). After dissecting the embryos out of extraembryonic membranes, the E-CSF was aspirated as previously described (Gato et al., 2004). To minimize protein degradation, E-CSF samples were kept at 4°C, aliquoted, lyophilized, and frozen at -40°C until used.

Organotypic Cultures of Mesencephalic Neuroectoderm

Chick embryos at HH20 stage were removed from the egg and placed in a Petri dish with sterile saline. After dissecting the embryos out of the extraembryonic membranes, the ectoderm covering the dorsal part of the mesencephalic vesicle was removed under dissecting microscope control using a thin tungsten needle, and the mesenchyme adhering to the explant were eliminated as far as possible by microdissection. We avoided doing this by enzymatic digestion so as to preserve the integrity of the extracellular matrix of the neuroepithelial tissue itself and its basal membrane, which might be important in affecting behavior. Nevertheless, the behavior of the explants cultured in a defined medium with no contribution from exogenous factors showed that the tiny fragments of mesenchyme adhering to the explants were unable to modify substantially the way neuroepithelial cells behave. The dorsal region of mesencephalon was then cut off with microscissors following a craneocaudal sectioning line that was dorsal to the diencephalon-mesencephalon fold and the mesencephalon-rhombencephalon isthmus (important organizing center); consequently, the explants comprised the roof plate of the midbrain and the neuroepithelium lateral to it, with a size ranging from 7 to 10 mm². During the sectioning of the explant, its edges stuck together, maintaining their basal-apical orientation; in addition, the roof plate differentiated from the rest of the neuroepithelium due to its greater transparency, permitting the explant's anterior-posterior orientation. Following our adaptation of Trowell's technique for organotypic tissue cultures (Brunet et al., 1993), the explants were transferred to a culture well containing a chemically defined serum-free medium (DMEM: F12, Sigma) supplemented with 1% ascorbic acid and were carefully washed three times in serum free-medium. Small rectangles of Millipore filters (0.8 μm pore size) previously boiled in distilled and deionized water were equilibrated in serum-free medium for 15 min, and the explants were placed on top of the filter with the apical surface of the neuroepithelium in close contact with the dark surface of the filter. To avoid the detachment of the explants, they were peripherally fixed to the filter with the tungsten needle, orienting the axis of the roof plate parallel to the large axis of the filter paper. Finally, they were cultured at 37°C with 5% CO₂ for 24 hr (which corresponded chronologically to stage HH23).

Cultured explants and control embryos were processed to monitor several parameters of neuroepithelial stem cell behavior, i.e., BrdU incorporation, apoptosis, and neuronal differentiation in various sets of experiments. In all cases, the study was made with histological samples taken from the central area of the explants to standardize results and to avoid damage to peripheral tissue during handling of the explants. The experiments were as follows.

First, to establish the normal pattern of BrdU incorporation, apoptosis and neuronal differentiation at the beginning and at the end of the period analyzed, control embryos were maintained *in vivo* until developmental stages HH20 or HH23. For BrdU incorporation, mesencephalic neuroepithelial explants obtained from embryos of developmental stages HH20 and HH23 were cultured *in vitro* in the presence of BrdU for 1 hr.

Second, to test the validity of the organotypic culture technique in the absence of other structures that surround the explant *in vivo*, mesencephalic neuroepithelial explants were cultured for 24 hr with a defined culture medium supplemented with 7% fetal calf serum (FCS).

Third, to test the developmental autonomy of the mesencephalic neuroepithelial stem cells compared with extraneural surrounding signals during the developmental stages analyzed, neuroepithelial explants were cultured for 24 hr with only a defined culture medium.

Fourth, to test the trophic role of the E-CSF in neuroepithelial stem cell behavior, neuroepithelial explants were cultured for 24 hr with a defined culture medium supplemented with E-CSF at 1/7 v/v. Although these organotypic cultures were prepared with mesencephalic neuroectoderm from embryos cultured *in vivo* until HH20 and left to develop for 24 hr (corresponding chronologically to stage HH23), the E-CSF was obtained from embryos at stage HH25 to obtain a reasonable amount of this fluid (approximately 5 μl/embryo). This temporal mismatch may not significantly affect the *in vitro* behavior of the mesencephalic neuroepithelial explants, as the protein composition of the E-CSF is not qualitatively modified between HH20 and HH25 (Gato et al., 2004).

Finally, to test the presence of diffusible molecules in the E-CSF affecting neuroepithelial stem cell behavior, heparin acrylic microbeads (100–200 μm diameter; Sigma) were soaked in E-CSF for 8 hr at 4°C. They were briefly rinsed in serum-free medium to remove excess E-CSF and placed between the filter paper and the explants. These neuroepithelial explants were cultured for 24 hr in a serum-free medium.

BrdU and β3-Tubulin Determination

Determination of BrdU incorporation into cell nuclei was performed by adding BrdU to the culture medium at a final concentration of 5 μM for 1 hr at the end of the organotypic culture. Immediately after this, the explants were fixed in Carnoy for 20 min, dehydrated in an alcohol series, passed through xylene, and embedded in paraffin. After cutting the tissues transversally to the roof plate, they were deparaffinized and BrdU was detected following standard procedures. The sections were incubated in a solution containing a monoclonal antibody to BrdU (Dako) at 1/100 for 30 min at RT. To detect the primary antibody, the avidin-extravidin system conjugated to peroxidase (mouse antirabbit 1/20 for 30 min and extravidin 1/20 for 10 min; Sigma) was used and staining was developed with DAB. For visualizing and photographing the preparations, we used a Nikon microphot-FXA photomicroscope. A quantitative analysis of nuclear BrdU incorporation was performed by counting the number of BrdU-positive nuclei in 34 microscopic fields of 1,400 μm², taken from the central region of each explant, and from 5 different explants. The average of each condition and the standard error were plotted, and their significance was tested by an unpaired two-tailed Student's *t*-test.

To detect early neuronal differentiation, we monitored β3-tubulin (Tuj-1) expression. After organotypic culture, the explants were processed as described below for BrdU detection but using a monoclonal antibody anti-β3-tubulin at 1/500 (BAbCO) and an antimouse antibody conjugated to FITC at 1/64 (Sigma) for 1 hr at RT. Controls were performed on histological sections of chick embryos at

developmental stage HH23. For visualization and photographing of the preparations, we used a confocal microscope (Zeiss LSM-310). A quantitative analysis of β 3-tubulin localization was performed by counting the number of neuroepithelial cells with immunostained cytoplasm in 20 microscopic fields of $1,900 \mu\text{m}^2$, taken from the central area of each explant, and from 4 different explants. The average of each condition and the standard error were plotted, and their significance was tested by an unpaired two-tailed Student's *t*-test.

TUNEL Assay

Apoptotic cells were detected by the TUNEL assay on sagittal paraffin sections from formalin-fixed sections. Apoptotic cells were detected using the Apoptosis Detection System Fluorescein Kit (Promega) following the manufacturer's instructions. Visualization was made with a confocal microscope (Zeiss LSM-310). We did not perform any quantitative analysis of apoptotic cells, as only the explants cultured in serum-free medium showed a great number of positive cells.

RESULTS

Organotypic In Vitro Tissue Culture of Neuroepithelial Explants

The technique of organotypic in vitro tissue culture of chick neuroepithelium explants allowed us to analyze the cellular behavior of the neuronal stem cells of the mesencephalic vesicle. To test the validity of this technique, we analyzed three parameters, apoptosis (by TUNEL assay), proliferation (by BrdU incorporation), and neurogenesis (by β 3-tubulin immunolabeling), in two sets of experiments: control embryos maintained in vivo and killed at the beginning (HH20) or at the end (HH23) of the period analyzed, and mesencephalic neuroepithelial explants cultured 24 hr from HH20 in FCS-supplemented medium.

The chick mesencephalic neuroepithelium of control embryos developed in vivo until stage HH20 or HH23 revealed very few apoptotic cells, scattered along this tissue both at the beginning (data not shown) and at the end of the period analyzed (Fig. 1A). These controls also showed a large number of BrdU-positive nuclei with a basal-apical pattern of distribution (Fig. 1E). Finally, the initiation of neurogenesis, monitored by β 3-tubulin immunostaining, was detected at the beginning of the period analyzed (i.e., HH20) as a faint cellular immunolabeling discontinuously distributed within the basal portion of the neuroepithe-

lium (Fig. 1J). β 3-tubulin immunostaining increased during the period analyzed. At HH23, β 3-tubulin immunoreactive cells were detected in a continuous layer at the basal portion of the mesencephalic neuroepithelium. These cells showed intense cytoplasmic labeling and some cytoplasmic processes running from the basal to the apical portion of the neuroepithelium (Fig. 1K).

To test the validity of the organotypic culture technique, neuroepithelial explants from the mesencephalon were cultured in vitro from HH20 in a defined medium supplemented with 7% FCS for 24 hr. After culture, the neuroepithelial cells of these explants behaved similarly to the neuroepithelial stem cells maintained in vivo (the controls), i.e., they showed very few apoptotic cells scattered along the neuroepithelium (Fig. 1B) and the amount of nuclei that had incorporated BrdU was very similar to that of the control specimens (Fig. 1F), which displayed the same basal-apical pattern of BrdU-positive nuclei distribution. Moreover, the number of BrdU-positive cells in controls and explants cultured with FCS-supplemented medium did not differ significantly (Fig. 2).

Likewise, the explants cultured with the FCS-supplemented medium showed the same pattern of neurogenesis, monitored by β 3-tubulin immunostaining, as the controls (Fig. 1L). However, the number of β 3-tubulin-positive cells was approximately 30% less than in the explants cultured with FCS-supplemented medium than in the controls, indicating a reduction in neurogenesis (Fig. 3). Despite this decrease, which is discussed below, our data endorse the validity of this organotypic tissue culture technique for neuroepithelial explants, as it is a very useful tool to study the behavior of neuroepithelial stem cells and to analyze the role of factors regulating the early development of the central nervous system.

Absence of Exogenous Trophic Factors Modifies Cellular Behavior of Neuroepithelial Stem Cells

To test whether the cellular behavior of neuroepithelial stem cells is autonomous or depends, at least partially, on stimuli other than those of the neuroepithelium itself, mesencephalic neuroepithelial explants were cultured with serum-free medium without the addition of any exogenous trophic factor. In this set of experiments, the cellular behavior of neuroepithelial stem cells largely differed from that of controls and the explants cultured with FCS-supplemented medium. In these cultures, abundant apoptotic cells scattered along the whole of the neuroepi-

Fig. 1. **A–D:** Apoptosis analysis by TUNEL assay on chick embryo mesencephalic neuroepithelium at developmental stage HH23 on embryos maintained in vivo (A) or after 24 hr of organotypic culture of mesencephalic neuroepithelial explants (from HH20) at various experimental conditions: (B) explant cultured with FCS-supplemented medium; (C) explant cultured with a chemically defined medium (DMEM-F12), and (D) explant cultured with E-CSF-supplemented medium. Arrows point to apoptotic cells. **E–I:** Cell proliferation analysis by BrdU incorporation on chick embryos mesencephalic neuroepithelium at developmental stage HH23 on embryos maintained in vivo (E) or after 24 hr of organotypic culture of mesencephalic neuroepithelial explants (from HH20) at various experimental conditions: (F) explant cultured with FCS-supplemented medium; (G) explant cultured with a chemically defined medium (DMEM-F12); (H) explant cultured with E-CSF-supplemented

medium; and (I) explant cultured with a chemically defined medium with an implanted heparin acrylic microbead previously soaked in E-CSF. **J–O:** Neural differentiation monitored by β 3-tubulin immunostaining on chick embryos mesencephalic neuroepithelium at developmental stages HH20 (J) or HH23 (K) on embryos maintained in vivo, or after 24 hr of organotypic culture of mesencephalic neuroepithelial explants (from HH20) at various experimental conditions: (L) explant cultured with FCS-supplemented medium; (M) explant cultured with a chemically defined medium; (N) explant cultured with E-CSF-supplemented medium; and (O) explant cultured with a chemically defined medium with an implanted heparin acrylic microbead previously soaked in E-CSF. a, apical side of the neuroepithelium; b, basal side of the neuroepithelium; hb, heparin acrylic microbead. Scale bar = 30 μm (A–D, J–N); 50 μm (E–I and O).

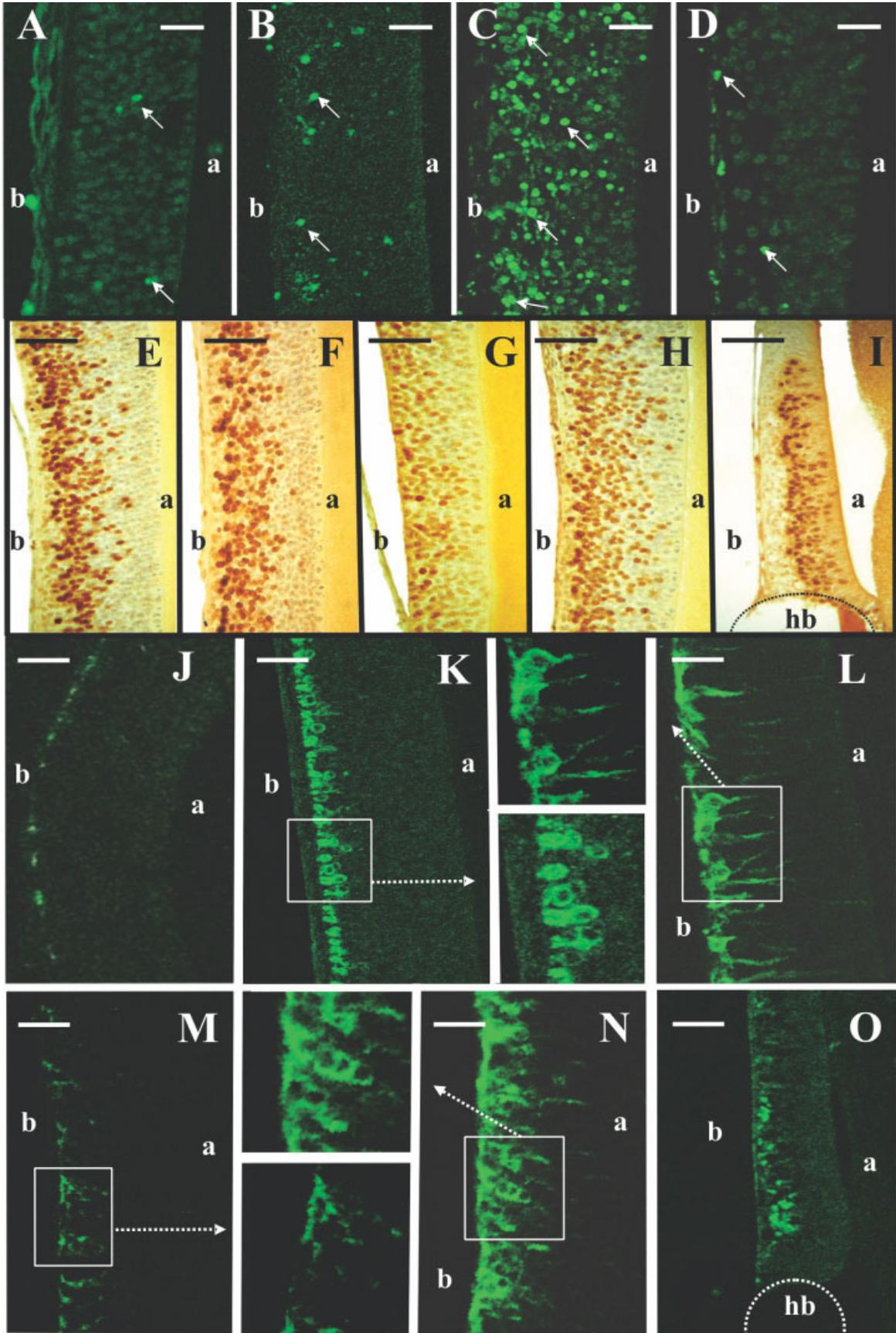


Figure 1.

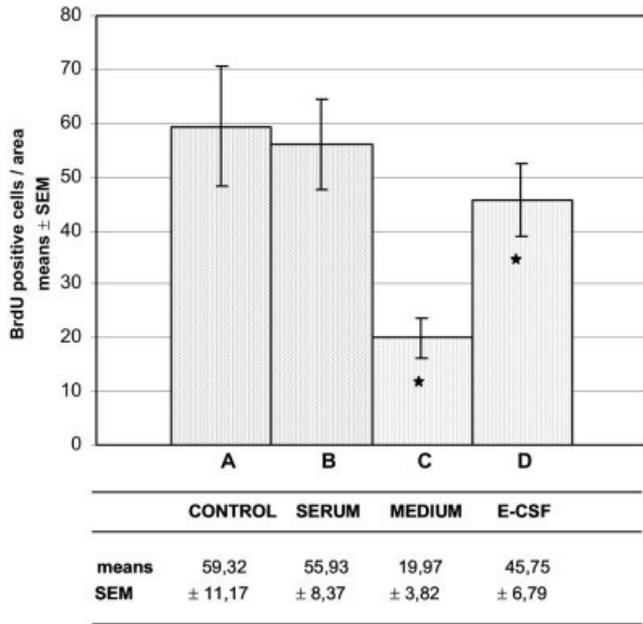


Fig. 2. Quantitative analysis of neuroepithelial cells synthesizing DNA measured by the number of BrdU-positive cells. Values plotted in the chart show the mean of the BrdU-positive cells per area \pm the standard error. A: Mesencephalic neuroepithelium of chick embryos maintained in vivo until developmental stage HH23. B: Mesencephalic neuroepithelium explants cultured in vitro with FCS-supplemented medium. C: Mesencephalic neuroepithelium explants cultured in vitro with a chemically defined serum-free medium. D: Mesencephalic neuroepithelium explants cultured in vitro with E-CSF-supplemented medium. Note that the number of BrdU-positive cells is similar in the controls (A) and in the explants cultured with FCS-supplemented medium (B). Also note that the number of BrdU-positive cells in the explants cultured with E-CSF-supplemented medium (D) is slightly lower than in A and B, and that in the explants cultured with serum-free medium (C), the number of BrdU-positive cells is much lower than in the rest of experimental conditions, including the explants cultured with E-CSF-supplemented medium (D). Asterisks denote values that differ significantly ($P < 0.05$) from controls according to the unpaired two-tailed Student's *t*-test.

thelium were detected (Fig. 1C), thus indicating a remarkable decrease in cell survival.

Moreover, the number of nuclei that had incorporated BrdU was strongly reduced, revealing significant differences from controls (Fig. 2), thus indicating a reduction in the number of cells synthesizing DNA, although the basal-apical pattern of distribution was preserved (Fig. 1G). In these cultures, some areas of the neuroepithelium were clearly thinner than in the controls or the explants cultured with FCS-supplemented medium (compare Fig. 1G with E and F). These results clearly indicate that neuroepithelial stem cells cannot maintain their normal rate of replication by themselves, suggesting that this process at least partially depends on the action of trophic factors other than those present or produced by the mesencephalic neuroepithelium.

These organotypic cultures also showed a drastic and significant decrease in β 3-tubulin immunostaining compared with the controls (Figs. 1M and 3), thus indicating that the differentiation process that drives neuroepithelial

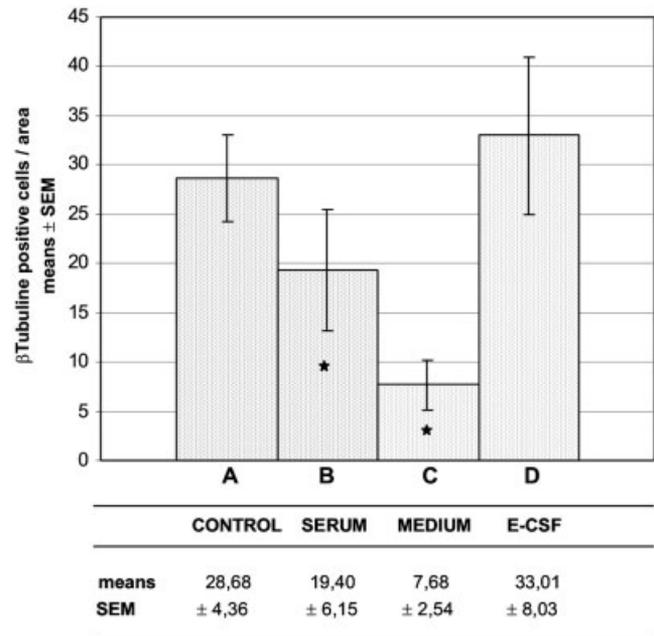


Fig. 3. Quantitative analysis of neuroepithelial cells undergoing neural differentiation measured by the number of β 3-tubulin-positive cells. Values plotted in the chart show the mean of the β 3-tubulin-positive cells per area \pm the standard error. A: Mesencephalic neuroepithelium of chick embryos maintained in vivo until developmental stage HH23. B: Mesencephalic neuroepithelium explants cultured in vitro with FCS-supplemented medium. C: Mesencephalic neuroepithelium explants cultured in vitro with a chemically defined serum-free medium. D: Mesencephalic neuroepithelium explants cultured in vitro with E-CSF-supplemented medium. Note that the number of β 3-tubulin-positive cells is slightly higher in the explants cultured with E-CSF-supplemented medium (D) than in the controls (A), although this difference is not significant; the explants cultured with serum-free medium (C) show a drastic reduction in β 3-tubulin-positive cells. Also note that in the explants cultured with FCS-supplemented medium (B), the number of β 3-tubulin-positive cells is slightly lower than in the controls and the explants cultured with E-CSF-supplemented medium. Asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's *t*-test.

stem cells to a neural fate may also require exogenous trophic factors.

Taken together, all these data suggest that the cellular behavior of neuroepithelial stem cells at early developmental stages depends, at least in part, on trophic factors other than those present or produced by the mesencephalic neuroepithelium.

Trophic Role of E-CSF in Neuroepithelial Stem Cells

At early developmental stages, all neuroepithelial stem cells are in close contact with the E-CSF, which fills up the cephalic cavities. As the cellular behavior of the neuroepithelial stem cells at these stages seems to depend on trophic factors other than those present or produced by the mesencephalic neuroepithelium, we checked whether the E-CSF conditions the behavior of these cells. Mesencephalic neuroepithelial explants cultured with serum-free medium supplemented with E-CSF obtained from

HH25 chick embryos did not undergo extensive apoptosis (Fig. 1D) as did the explants cultured only with serum-free medium. On the contrary, apoptotic cells were scarce, as detected in controls. These data suggest that the E-CSF promotes the survival of these cells.

Moreover, the addition of E-CSF to the serum-free medium in these cultures largely prevents the drastic decrease in nuclear BrdU incorporation shown by explants cultured with only serum-free medium (compare Fig. 1G and H). The number of BrdU-positive nuclei in the neuroepithelial explants cultured in the E-CSF-supplemented medium was slightly lower than that in controls. Although this difference was almost double, the number of BrdU-positive nuclei was clearly higher than that of explants cultured in serum-free medium (Fig. 2).

Subsequently, to test whether the role of the E-CSF in cell replication is due to the presence of diffusible factors within it, we implanted heparin acrylic microbeads soaked in E-CSF in neuroepithelial explants cultured in serum-free medium. Abundant BrdU-positive nuclei were located in the basal portion of the areas of the neuroepithelium that were close to the microbead (Fig. 1I). In this way, the number of BrdU-positive cells progressively decreased from the microbead outward, suggesting the presence of diffusible factors within the E-CSF, which may be involved in the control of neuroepithelial cell replication.

Finally, the addition of E-CSF to the serum-free medium in these organotypic cultures also induced the expression of β 3-tubulin in a continuous layer of cells located at the basal portion of the mesencephalic neuroepithelium, and some of the β 3-tubulin-positive cells showed long cytoplasmic developments from the basal to the apical part of the neuroepithelium (Fig. 1N), as shown in the controls. The number of β 3-tubulin-positive cells present in the neuroepithelial explants cultured with E-CSF-supplemented medium was similar to or slightly higher than in the controls, although this difference was not significant (Fig. 3).

Finally, to determine whether the role of the E-CSF in neural differentiation is due to the presence of diffusible factors within it, we also implanted heparin acrylic microbeads soaked in E-CSF in neuroepithelial explants cultured in serum-free medium. Abundant β 3-tubulin-positive cells were detected in areas of the neuroepithelium close to the microbead. Moreover, the number of β 3-tubulin-positive cells progressively decreased from the acrylic microbead outward (Fig. 1O), suggesting the presence of diffusible factors within the E-CSF, which may be involved in neurogenesis.

Taken together, all these data indicate that the E-CSF contains diffusible factors that regulate the three basic cellular behavioral parameters of the neuroepithelial stem cells analyzed in this study, i.e., survival, replication, and differentiation, suggesting that the E-CSF could play a key role in early brain development *in vivo*.

DISCUSSION

We have developed an organotypic tissue culture technique for neuroepithelial explants that allows us to study the influence of external factors on cellular behavior. We have shown the validity of this technique, as the cellular behavior of the neuroepithelial cells of mesencephalic explants cultured in FCS-supplemented medium was very similar to that of controls kept *in vivo*, i.e., the trophic

factors within the FCS are sufficient to ensure an almost normal rate of cell survival, proliferation, and differentiation. Despite the fact that the behavior of neuroepithelial cells *in vivo* differs somewhat from that observed in cultures with an FCS-supplemented medium, these differences could be due to the downtime that all tissues take to adjust to the trauma, *in vitro* conditions, etc. We have also demonstrated that mesencephalic neuroepithelial explants cultured *in vitro* in serum-free medium cannot maintain the normal rate of cell survival, proliferation, and differentiation by themselves, but that the addition of E-CSF to a serum-free medium is also sufficient to maintain almost normal cellular behavior compared with controls *in vivo*. These results clearly suggest that E-CSF exerts a trophic effect on mesencephalic neuroepithelial cells *in vivo*, having a direct influence on the behavior of neuroepithelial cells.

Regulation of Cellular Behavior of Neuroepithelial Cells Requires Extraneural Factors

Neuroepithelial cells undergo intense cell proliferation and simultaneously begin neurogenic differentiation (Panchision and McKay, 2002). Several growth factors have been involved in the control of these processes, including FGF2 and EGF (Murphy et al., 1990; Ciccolini and Svendsen, 1998; Tropepe et al., 1999; Vaccarino et al., 1999a, 1999b; Raballo et al., 2000), which are expressed together with their receptors by the neuroepithelial cells from early stages of development and act in an autocrine and/or paracrine fashion (Heuer et al., 1990; Kalcheim and Neufeld, 1990; Ozawa et al., 1996; Wilke et al., 1997; Raballo et al., 2000; Panchision and McKay, 2002).

However, it has also been suggested that there are other behavioral influences different from neuroepithelial self-stimulation (Kalcheim and Neufeld, 1990; Miyan et al., 2003). In this regard, Gato et al. (1998) and Raballo et al. (2000) have suggested that FGF2 is also secreted to the E-CSF, and that it may help regulate neuroepithelial cell behavior, interacting with apical receptors. Moreover, an anti-FGF2 antibody introduced by microinjection into cephalic ventricular cavities *in vivo* impairs cell replication and neurogenesis in the subventricular region (Tao et al., 1997), which is the fetal and adult successor of embryonic neuroepithelial cells (Bruni, 1998; Tramontin et al., 2003). These findings support the hypothesis that the CSF is an alternative fluid way to control the cellular behavior of the cells that are in close contact with brain cavities (Tao et al., 1997; Gato et al., 1998, 2004; Vaccarino et al., 1999a, 1999b).

Therefore, our data show that the cell proliferation and neurogenesis in neuroepithelial explants cultured with serum-free medium clearly decreased compared with controls, and that the apoptosis rate greatly increased. Moreover, the addition of the growth factors, present in the FCS, to the culture medium allowed the explants to maintain almost normal cell behavior. These results suggest that the neuroectoderm needs a supplementary contribution of extraneural growth factors, in addition to autocrine and/or paracrine neuroepithelial secretion, to ensure normal neuroepithelial cell behavior *in vivo*. Although these factors may come from the neural tissues that surround *in vivo* the explanted area (e.g., the midbrain-hindbrain isthm-

mus), we cannot rule out the possibility of a supplementary extraneural source of growth factors *in vivo*, which could be extraneural tissues such as the notochorda or the E-CSF itself.

E-CSF Helps Control Cellular Behavior in Neuroepithelial Cells

Classically, E-CSF has been involved in some epigenetic processes during early brain development, e.g., the positive pressure that drives expansion and morphogenesis (Jelinek and Pexieder, 1968, 1970; Desmond and Jacobson, 1977), which is regulated by apical secretion of osmotically active molecules belonging to the family of proteoglycans (Gato et al., 1993; Alonso et al., 1998, 1999).

On the other hand, E-CSF has a complex protein composition, and the exposure of the apical surface of neuroepithelial cells to this fluid could affect their behavior (Dziegielewska et al 1980a, 1980b, 1981, 1991, 2000; Checui et al., 1984; Gato et al., 2004). Experimental data demonstrate that the loss or modification of the composition of the E-CSF strongly decreases the neuronal cell population (Desmond and Jacobson, 1977; Desmond, 1985). Moreover, Mashayesky et al. (2002) and Owen-Lynch et al. (2003) have reported that the phenotype of the mutant hydrocephalic Texas rats is due to an alteration of the proliferation of brain primordium stem cells and cortical neurogenesis due to changes in the composition of the CSF. In this regard, the trophic action of CSF from rat fetus on cortex neuroblastic cells cultured *in vitro* has been demonstrated (Miyan et al., 2003). Here we provide experimental evidence that neuroepithelial cell survival, replication, and differentiation are activated by E-CSF in organotypic cultures, suggesting that the E-CSF contains key factors involved in the control of these cellular processes and, consequently, plays a relevant role in the development of the embryonic brain *in vivo*. Our results show that cell replication (but not neurogenesis) is slightly but significantly lower in the explants cultured with E-CSF than in the controls developed *in vivo*, suggesting that other factors from neural or extraneural tissues could be involved in maintaining the normal rate of neuroepithelial cell replication.

Finally, in the past years it has been suggested that adult CSF plays a key role as a fluid way to deliver diffusible signals and thus influence the cellular behavior of determined brain parenchyma cells (Nicholson, 1999). More recently, Alvarez-Buylla and García-Verdugo (2002) and Tramontin et al. (2003) have demonstrated that in the adult brain, the neurogenic stem cells of the subventricular zone have transitory contact with the ventricular brain cavity, suggesting that adult neurogenesis could be conditioned by the CSF.

How Does E-CSF Control Cellular Behavior of Neuroepithelial Pluripotent Stem Cells?

We have reported that the E-CSF could play a relevant role in the early development of the brain, in addition to other morphogenetic mechanisms (expansion of the brain vesicle cavities). As stated above, several studies have demonstrated that the protein composition of the E-CSF is more complex than that of the adult CSF and it has been suggested that there exist proteins involved in the regulation of cell behavior, e.g., growth factors and cytokines.

Moreover, it has also been suggested that there exist various growth factors in chick embryo brain cavities (Gato et al., 1993; Ojeda and Piedra, 2000). In this regard, we have previously detected the presence of FGF2 in the E-CSF of chick embryos at early stages of development (Gato et al 1998). We are currently analyzing the presence of several growth factors and cytokines in the E-CSF, as well as their role in neuroepithelial stem cell behavior *in vivo*.

Regarding the origin of the diffusible factors within the E-CSF during early developmental stages, prior to the formation of the choroids plexus, this fluid may be secreted at least partially by the neuroepithelial cells themselves (Miyan et al., 2003). However, the protein composition of the E-CSF is very similar to embryonic serum at the same developmental stages (Gato et al., 2004), which suggests that at least some of the proteins within the E-CSF could gather and be selectively transported from the embryonic serum to the E-CSF.

Perspectives

To conclude, this study supports the idea that early histogenesis of the embryonic brain might not be an intrinsic process of the neuroepithelium, but one requiring the action of external factors, some of which act from the E-CSF. Our data support the hypothesis that the fluid within the cavity of the embryonic brain is a key pathway for the diffusion of molecular signals *in vivo*. This implies that a complete understanding of the process of embryonic neurogenesis requires further analysis of the molecules within the E-CSF and their role in neurogenesis. These data may also improve our knowledge of the cellular requirements for neuronal culture techniques and allow us to extrapolate them to the regulation of neurogenesis in adults.

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