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Short Report

Trophic effect of cerebro-spinal fluid on primitive neuroepithelial cells in chick embryos

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One of the first signs of morphological differentiation in the embryonic brain takes place early in development with an explosive increase in the volume of the anterior end of the neural tube principally due to an increase in cavity size. During this expansive process the neural tube is formed by an epithelial wall named neuroepithelium and a great cavity containing the primitive cerebro spinal fluid (CSF). In earliest stages basically neuroepithelial cells undergoes three process which are proliferation, migration and differentiation. It has been shown that CSF are responsible of the generation of the mechanical forces involved in embryonic brain expansion (Alonso *et al.,* 1998)), furthermore this fluid has a complex proteic composition (Fielitz *et al.,* 1984) and we have demonstrated that fibroblast growth factor 2 (FGF-2) are a component of this fluid which exert an influence in neuroepithelial cells replication (Gato *et al.,* 1998). To date the influence of the CSF on neuroepithelial cells behaviour remains unclear, so the aim of this work is to determinate if CSF has a role in proliferation, survival and differentiation of neuroepithelial cells during earliest stages of development in chick embryos.

For our study we have obtained CSF by mesencephalic microaspiration of 21 and 24 H.H. (Hamburger and Hamilton 1951) stages of chick embryos, and it was employed immediately after extraction.

Neuroepithelial *"in vitro"* culture was a modification of Trowell´s culture, briefly, neuroepithelial squares of 21 H.H. stage embryos were carefully dissected from the lateral mesencephalic vesicle after ectoderm resection then they were carefully rinsed in sterile Ringer solution to eliminate the CSF residues. The neuroepithelial samples were laid by the apical side onto Millipore filters in contact with 300 μl of DMEM/F12 medium (Sigma) supplemented with 1% of Ascorbic acid and incubated at 37.5°C in a 5% CO2 atmosphere during 24 hours. We have carried out four different experimental conditions: 1.- Cultures only with defined medium. 2.- Cultures with medium and 10 μl of CSF. 3.- In several cultures we have employed 100-200 μm latex microbeads soaked in CSF for 2 hours and placed between the neuroepithelium and the millipore filter. 4.- As controls we have used mesencephalic neuroepithelium taken out from alive embryos at the same developmental stages. Samples from each one of these experimental conditions were fixed in Carnoy, dehydrated in alcohol and embedded in paraplast and then processed for proliferation, apoptosis and differentiation studies. Neuroepithelial cell proliferation was measured by BrdU incorporation, for this purpose one hour before of the end of the culture we added to the medium 10 μl of a 3% solution of BrdU (Sigma) in PBS, BrdU incorporated to the nucleus was detected by immunolabelling with a monoclonal anti-BrdU antibody (Dako) and a secondary antibody labelled with Extravidin-Peroxidase. To localise apoptosis we employed the TUNEL method with an Apoptosis Detection System Fluorescein Kit (Promega) according to the manufacturer's recommendations. Neuroblasts differentiation were detected by βTubulin (βIII) expression recognised by a specific monoclonal antibody (BAbCO) and a FITC conjugated secondary antibody. For visualisation and photographing of the preparations we have used a confocal microscope (ZEISS LSM-310) and a Nikon microphot-FXA photomacroscope.

Our results shown that mesencephalic neuroepithelium in control living embryos only presented scarce and disperse apoptotic cells (Data not show), similar results were obtained in medium supplemented with CSF cultures (Fig. 1B). Nevertheless the neuroepithelium cultured in defined medium (without CSF) shown a great amount of apoptotic nucleus preferentially located at the basal side (Fig. 1A). In this area in controls appeared differentiated neuroblasts (Fig. 2A).suggesting that CSF has neurotrophic factors involved in postmitotic neuroblasts survival.

The immunolabelling of β III tubulin in at 23-24 H.H. stages control alive embryos was positive in a single file of cells near the basal surface of the neuroepithelium which will form the marginal layer (Fig. 2A). The presence of β III tubulin in neuroepithelial cells point out the existence of neuroblastic differentiation at these stages of development. Neuroepithelial cultures without CSF (Fig. 2B) did not show any evidence of β III tubulin expression, whereas the addition of CSF to the culture medium induced a similar β III tubulin immunomarking (Fig. 2C) than in control alive embryos in several cells layered near to the basal end of the neuroepithelium. On the other hand we have proved by implantation of latex microbeads soaked in CSF a local β III tubulin expression more intense in the cells around the microbeads and a decrease in the periphery (Fig. 2D). These data suggest that CSF has a direct influence in neuroblastic differentiation, and this effect could be produced by diffusible substances like growth factors or cytokines presents in the CSF.

Proliferation study shown that in control alive embryos there were a great number of BrdU positive nucleus grouped as aggregates nearest to the basal end of the mesencephalic neuroepithelium, whilst that the more apical portion only presented nuclear mitosis without BdrU incorporation (Fig. 3C). In neuroepithelial cultures without CSF there was a dramatic reduction in the

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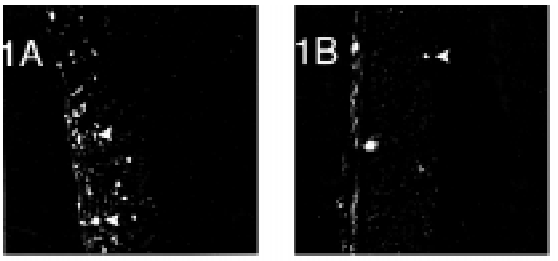

Fig.1. Apoptotic studies in neuroepithelium culture. *After 24 h of culture in defined medium, the neuroepithelium shows a lot of apoptotic nuclei (arrows) especially in the basal end* **(A)**. *When CSF was added to the culture medium, this apoptosis was mostly prevented* **(B)**.

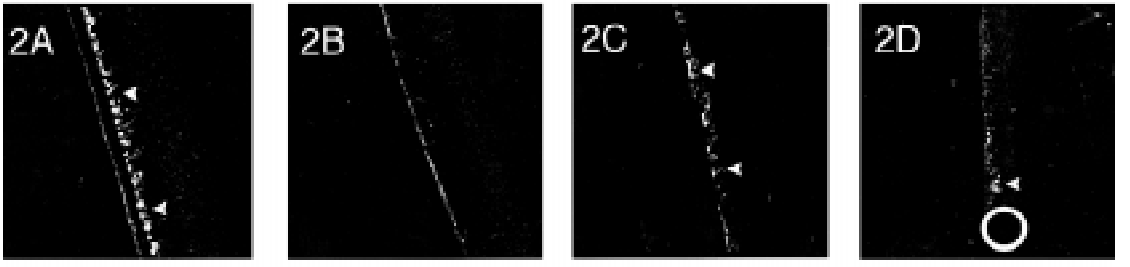

Fig. 2. β-III Tubulin expression in 24 H.H. embryo epithelium. *In live, control embryo neuroepithelium, a layer of neuroblast differentiated cells (β-III Tubulin positive) appeared in the basal end* **(A)** *(arrows). Neuroepithelium cultures in defined medium did not shown any β-III Tubulin expression* **(B)**. *However when CSF was added to the medium, a layer of β-III Tubulin positive cells (arrows) appeared in the same place as in the controls* **(C)**. *The presence of CSF-impregned latex beads (circle) induced β-III Tubulin expression (arrow) in the nearest neuroepithelial cells* **(D)**.

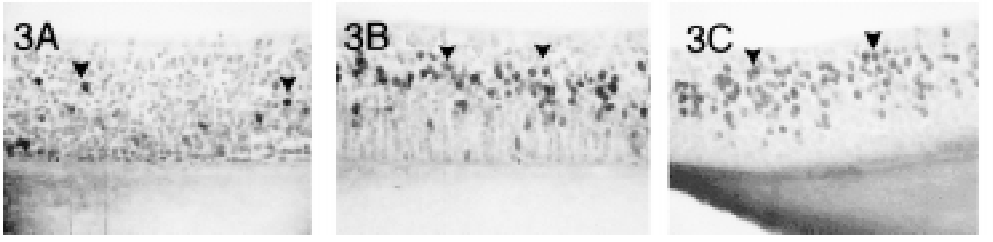

Fig. 3. Nuclear BrdU incorporation study. *In 24 H.H. stage live, control embryo neuroepithelium, there is a great number of BdrU positive nuclei (arrows) nearest to the basal end* **(C)**. *Neuroepithelium cultured without CSF* **(A)** *only showed scarce BrdU positive nuclei (arrows) and addition of CSF to the culture medium* **(B)** *produced a considerable increase in the number of labelled nuclei.*

number of BrdU positive nucleus along the entire mesencephalic wall (Fig. 3A). The addition of CSF to the culture medium shown to have a noticeable effect in neuroepithelial cells proliferation increasing considerably the number of BrdU positive nucleus most of them located as in control embryos (Fig. 3B). These results reveals that CSF could play a key role in neuroepithelial cells proliferation probably mediated by the presence of growth factors as FGF-2 which has been previously demonstrated by us (Gato *et al.,* 1998). Furthermore it is known that FGF-2 is the principal mitogen for undifferentiated neuroepithelial cells (Tao et *al.,* 1997), and these cells express apical FGF-receptors (Heuer *et al.,* 1990).

In conclusion, this study points out an interesting question as is that the neuroepithelial behaviour at earliest stages of development could be strongly influenced by the primitive CSF regulating the survival, replication and differentiation of the cells, probably by the presence of cytokines and/or growth factors in their composition.

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