

**Effect of e-CSF on morphological changes of neurosphere derived astrocytes**Siamak Yari <sup>1</sup>, Kazem Parivar <sup>1</sup>, Mohammad Nabiuni <sup>1</sup>, Mohammad Keramatipour <sup>2</sup><sup>1</sup> Faculty of Biological sciences, Kharazmi University, Tehran, Iran<sup>2</sup> Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran  
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**Abstract:** Cultured astrocytes generally display flat polygonal shape but morphological change can be induced by some agent. It is well known that embryonic cerebrospinal fluid have many important role in embryonic brain development. In the present study, we examined possible developmental changes induced by embryonic cerebrospinal fluid stimulation. Cultured neurosphere derived astrocytes prepared from the subventricular zone of embryonic day 15.5 (E15.5) rat. Cultures treated with embryonic cerebrospinal fluid obtained from different embryonic ages (E16-E20). RT-PCR analysis shows that E17 and E18 CSF significantly increased the level of GFAP mRNA. Treatment with e-CSF induced stellation and convert polygonal astrocytes to process-bearing cells. Measurement of process length show significant increase in cultured treated with E16 and E18 but not in E20 CSF. Also, soma size in cultured exposure to CSF from E18 increased compare with control. The present study demonstrated that e-CSF have differential effect on morphological changes of neurosphere derived astrocytes.

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**1. Introduction**

Astrocytes are the most abundant cell type in the central nervous system (CNS). Astrocytes play many important roles in normal brain function. For example, astrocytes help to maintain homeostasis in the extracellular fluid (Walz, 1989), modulate neuronal excitability and synaptic transmission (Smith, 1992), provide trophic support for neuronal growth (Furukawa et al, 1986) and protect neurons from oxidative stress (Desagher et al, 1996). In addition, changes in morphology of astrocytes occur during brain development (Fedoroff, 1986). Astrocytes and their processes are supposed to guide migration and axonal growth of neurons during development (Mason et al, 1988). Astrocytes may also participate in establishing the blood-brain barrier (Beck et al, 1986).

Factors influencing the morphology of astrocytes have been widely studied in cell culture. Primary cultures of astrocytes generally exhibit flat polygonal shape, however, morphological changes can be induced by a variety of factors, such as co-culturing with neurons, exposure to reagents that increase intracellular cyclic AMP (Hatten, 1985; Goldman and Abramson, 1990) and exposure to  $\beta$ -amyloid peptide (Pike et al, 1994). Therefore, the study of factors regulating astrocyte morphology is of importance for understanding brain development.

An important neuroprogenitor germinal region, the subventricular zone (SVZ), is in close contact with cerebrospinal fluid (CSF) throughout embryonic period to adulthood. This proliferative

population of cells produces the majority of neurons and glial cells. Embryonic cerebrospinal fluid (e-CSF) secretion initially starts with the appearance of a special vascularized structure, the choroid plexus. Choroid plexus development initially occurs in lateral ventricular of 14 day old embryonic rat (Dziegielewska et al, 2001). Formation of choroid plexus and secretion of CSF within ventricles results in brain expansion in E15 rat that is responsible for proper brain development (Desmond and Jacobson, 1977).

Proteomic analysis of e-CSF has revealed the presence of many functional proteins and morphogenes as well as similarities in their composition between human and rat (Zappaterra et al, 2007). Furthermore, several studies have suggested that in this critical period of embryonic brain development, the protein profile present in the CSF has an important role in regulating the neuroprogenitor cells' behavior (Parada et al, 2006).

In the present study, we have examined the effect of e-CSF, obtained from different embryonic ages, on the morphological changes of neurosphere derived astrocytes.

**2. Material and Methods**

Wistar rats were bred in house in the research facility of the Department of Biology, Kharazmi University following ethical review of the project by the animal uses committees of Kharazmi University. They were kept in large rat boxes at a constant temperature with a 12 hour light/dark cycle and a free access to food and water. Individual male

and female rats were paired in mating cages and checked regularly for the presence of a vaginal plug which was taken as an indication of successful mating and the day noted as embryonic day 0 (E0). Embryonic age was calculated from that day. At a particular time point pregnant dams were euthanized by intraperitoneal injection of an overdose of sodium pentobarbitone, the uterus rapidly removed onto ice and fetuses dissected out onto ice. Each pregnant dam usually produced between 10–15 fetuses.

CSF was collected from the cisterna magna of rat fetuses at E16, E18, and E20 using glass micropipettes and capillary action without aspiration. Aspiration invariably resulted in bleeding and contamination of the samples. Fetuses were positioned with heads flexed down onto the chest to allow penetration into the cisternal cavity through the skin and underlying muscle. Samples containing undesirable blood contamination, visualized as a pink colour in the fluid, caused by damaging a blood vessel within the cisternal cavity, were discarded. All samples were collected into sterile microtubes and centrifuged at 1000 rpm to remove cells or debris from the fluid, and the supernatant was transferred into another sterile tube. These samples were stored at  $-80^{\circ}\text{C}$  until use. The volume of CSF collected from each fetus by this method was between 5 and 50  $\mu\text{l}$  and samples were pooled for each experiment.

Neurosphere were prepared as described previously (Reynolds and Weiss, 1992). Briefly, pregnant Wistar rat of gestational age 15.5 (E15.5) were killed via intraperitoneal injection of an overdose of sodium pentobarbitone. Embryos were removed from the amnion, and the heads were dissected using tweezers. After removal of the overlying meninges and blood vessels, subventricular zone (SVZ) was dissected out and transferred to serum-free media. Tissue samples were dissociated using 3 ml of 0.25% trypsin-EDTA (Gibco-Invitrogen) at  $37^{\circ}\text{C}$ , followed by a 5 ml trypsin inhibitor treatment. Cells were centrifuged at 1000 rpm for 5 minutes and resuspended in 5 ml of trypsin inhibitor and mechanically dissociated with a fire-polished pipette. Cells were then resuspended in 5 ml of basal media, centrifuged, resuspended in 2 ml of basal media and then used for the experiments. The cells were added to 25  $\text{cm}^2$  flasks and maintained in serum-free media comprising DMEM/F-12 medium (Gibco-Invitrogen) supplemented with 2% N2 (Gibco-Invitrogen) supplement, 1% penicillin/streptomycin (Gibco-Invitrogen), 20 ng/ml epidermal growth factor (EGF) (Gibco-Invitrogen) and 20 ng/ml basic fibroblast growth factor (b-FGF) (Gibco-Invitrogen). Cultures were incubated at  $37^{\circ}\text{C}$  in a humidified chamber with 5%  $\text{CO}_2$ . Fresh

medium and growth factors were supplemented every 2 days.

Cultures were divided into following study groups: 1. Control: No CSF exposure. 2. E16: Exposure to CSF of E16. 3. E18: Exposure to CSF of E18. 4. E20: Exposure to CSF of E20. For differentiation studies, after 4 days *in vitro*, cells obtained by neurosphere spilliting were counted and seeded onto poly-L-lysine (Sigma-Aldrich) coated surfaces and grown as a monolayer in the same medium without mitogens in order to allow them to differentiate. After 4 days *in vitro* (DIV) cells were processed by immunocytochemistry in order to perform antigen expression and morphological analysis.

To reveal the morphological changes in cultured neurosphere derived astrocytes, immunostaining was performed to confirm the expression of GFAP. Cells were fixed for 20 minutes in 4% paraformaldehyde in PBS (pH 7.4), washed in PBS and permeabilized for 5 minutes with PBS/0.5% Triton X-100 (Sigma-Aldrich). Adherent single cells were incubated overnight at  $4^{\circ}\text{C}$  in PBS containing 5% BSA and the appropriate mixture of antibodies. Primary antibody used was rabbit polyclonal anti-GFAP (1/600, Abcam) for astrocytes. After washing in PBS, differentiating cells obtained from spilled neurosphere were incubated for 1 hour with Cy3-conjugated secondary antibodies (1/300 Abcam). Nuclei were counterstained with propidium iodide (1/15000, Sigma-Aldrich), and morphological changes were observed using a microscope. Stellate astrocytes were defined as cells with one or more processes longer than their cell bodies. The total number of cells and the number of stellate cells were counted, and the percentage of stellate cells was determined by counting  $> 100$  cells in multiple fields.

Total cellular RNA was extracted using TRIZOL Reagent and the synthesis of cDNA and PCR was carried out using one-step RT-PCR kit (Invitrogen) according to the manufacturer's protocol. Total RNA (1  $\mu\text{g}$ ) was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas) and 1  $\mu\text{g}$  cDNA was subjected to PCR analysis using the following primer pairs: *Gapdh* forward, 5' -AGTGCCAGCCTCGTCTCATAG -3' ; *Gapdh* reverse, 5' -TAGACTCCACGACATACTCAGCAC -3' ; *Gfap* forward, 5' -CCACCAGTAACATGCAAG -3' ; *Gfap* reverse, 5' -CTTAACGTTGAGTAGATCCTG- 3'. The samples were analyzed on a 1% agarose gel containing ethidium bromide.

### 3. Results

Cultured neurosphere derived astrocytes in the presence of fetal bovine serum normally appeared

in flat, polygonal shapes without processes (Fig. 1a). When cells were exposed to e-CSF from different embryonic ages, they changed into stellate shapes bearing several processes in cultures conditioned with e-CSF from E16, E18 and E20. Exposure to E16 CSF, E18 CSF and E20 CSF significantly induced morphological changes. In the absence of stimulation (control cultures only supplemented with serum); cultured astrocytes obtained from E16 exhibited a flattened polygonal morphology (Fig.1&2).

We determined whether the gene encoding GFAP is differentially expressed during embryonic development. Semi-quantitative RT-PCR analysis revealed that the mRNAs encoding GFAP were expressed at comparable levels in cultures conditioned with E16, E18 and E20 CSF. However, GFAP mRNA was increased in astrocytes exposed to E18 CSF and decreased in cultures conditioned with E20 CSF (Fig. 5).

We determined the effect of e-CSF from different embryonic ages on astrocytes. Data show that in culture conditioned with e-CSF from 16 and 18 day old rat, an increased was seen in process length emanating from somata (Fig.1&4).

We assessed the effect of e-CSF on astrocytic soma size. astrocytic soma diameter measured by image j. astrocytes in cultures conditioned with CSF obtained from 18 day old embryonic rat have significant increase in soma size compare with control group but in cultures conditioned with CSF from E16 and E20 no obvious increase detected in soma size (Fig.1&3).

#### 4. Discussions

In the present study, we have demonstrated for the first time that e-CSF from different embryonic ages induce stellation of embryonic rat astrocytes, and that these effects on GFAP expression are developmentally regulated.

During embryonic development of the rat cortex, neurons and glial cells are generated in distinct phases: an early phase with neuronal differentiation peaking around E12–E15, and a late phase with astroglialogenesis predominantly beginning at approximately E16 and ending post-natally. A number of mechanisms, both intrinsic and extrinsic, have been proposed to contribute to this orderly differentiation program which ensures no premature astroglialogenesis during the neurogenic period, while maintaining a timely switch to astroglialogenesis following the completion of neurogenesis (Ross et al, 2003; Miller and Gauthier, 2007). For example, BMP, bFGF and EGF have been implicated in the regulation of the neurogenic to gliogenic switch.

Recent studies have suggest that e-CSF, as an extrinsic environment, provides a number of morhogenes and growth factors such as BMP, bFGF

and EGF that play a key role in the fate of neuroprogenitor cells (Buddensiek et al, 2010).

Since evidence suggests that astrocyte processes guide migration and axonal growth of brain neurons (Mason et al, 1988), astrocyte stellation may thus contribute to the formation of neural network during brain development. In agreement with this result, we show that the effect of e-CSF on GFAP mRNA expression coincides with *in vivo* developmental pattern, and this expression begins in a culture conditioned with E16CSF. Therefore, we suggest that e-CSF could have a critical role in determining the timing of neural network formation by regulating the astrocyte morphology.

Evidence suggests that e-CSF contains retinoic acid (Parada et al, 2008), and that enzymes involved in the synthesis of RA have been identified in meninges and choroid plexus (Siegenthaler et al, 2009). Previous work has shown that RA, as a signaling homone, plays an important role in the differentiation of neuroprogenitor cells both *in vivo* and *in vitro* (Ribes et al, 2006). Moreover, retinoic acid has opposing effects on astroglialogenesis in cortical progenitors derived from early versus late developmental stages. Retinoic acid promotes astroglialogenesis during the late embryonic period, for example at E18, retinoic acid indused swith from neuronal fate to glial fate (Faigle et al, 2008). These findings indicate the possibility that e-CSF effects on morphological features of astrocytes are mediated by the RA present in CSF.

Evidence suggests a relationship between intracellular pH and the morphological changes of cultured astrocytes. Indeed, replacement of astrocytes to media lacking bicarbonate results in a decrease in intracellular pH followed by a rapid stellation (Cechin et al, 2002). In addition, astrocyte stellation has been found to be ECM-dependent. In fact, cultured astrocytes, similar to other cells such as fibroblast and epithelial cells, are influenced by physiological ECM, and exhibit a higher intracellular pH in response to these physiological matrices (Gottfried et al, 2003). Since, many physiological matrices such as fibronectin and laminin were present in embryonic CSF (Zappaterra et al, 2007). Consequently, we concluded the possibility that astrocytes stellation in cultured treated with e-CSF enhanced by ECM content of e-CSF.

Recent findings suggest that intermediate filaments of astrocytes such as GFAP and vimentin increase the reactive astrocyte process length and resulted in increased regeneration rate in CNS injury (Wilhelmsson et al, 2004). Our results show that E17 and E18 CSF have increased the process length of astrocytes which is in agreement with those findings that expression of GFAP mRNA increased in that

time point. So we concluded that elevated levels of GFAP expression may involved in astrocytic process length.

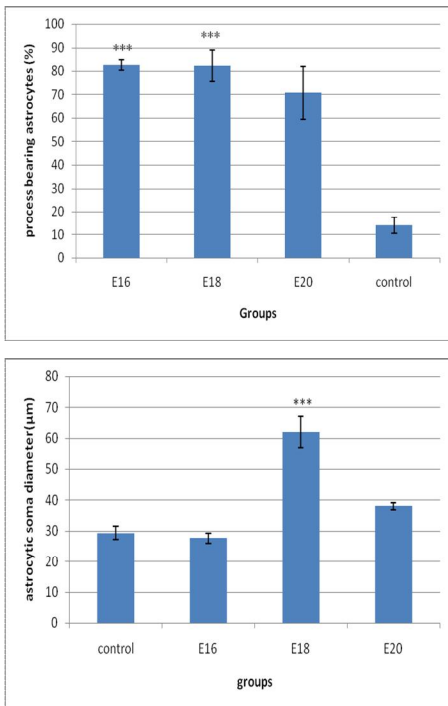


Fig 1: representative photomicrographs showing e-CSF induced morphological changes of cultured rat neurosphere derived astrocytes. Embryonic rat astrocytes(E15.5) were exposed to e-CSF from different embryonic periods(a:control, b: astrocytes exposed to E16-CSF, c: astrocytes exposed to E18-CSF and d: astrocytes exposed to E16-CSF.

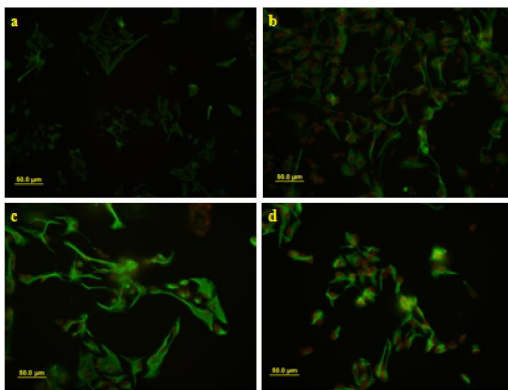


Fig 2: e-CSF stimulated stellation in rat embryonic astrocytes. The cells were exposed to CSF from E16, E18 and E20. The numbers of polygonal and stellate cells were counted and the percentages of stellate cells calculated. Data are means±S.E.M. (\*\*p<0.01& \*\*\*p<0.001).

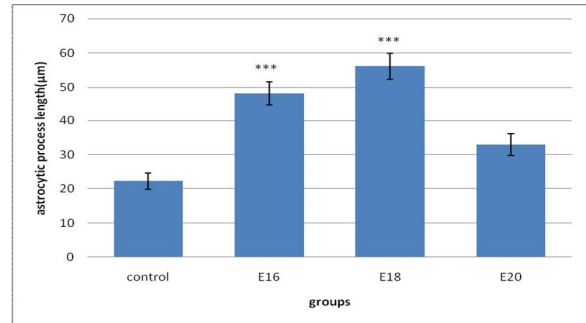


Fig 3: The effect of e-CSF on astrocytic soma size. Statistically significant differences between cultures treated with E18 CSF were detected compared with control group. Data are means±S.E.M. (\*\*p<0.01).

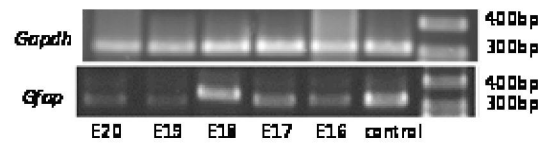


Fig 4: The effect of e-CSF on astrocytic process length. Statistically significant differences between cultures treated with E16 and E18 CSF were detected compared with control group. Data are means±S.E.M. (\*\*p<0.01).

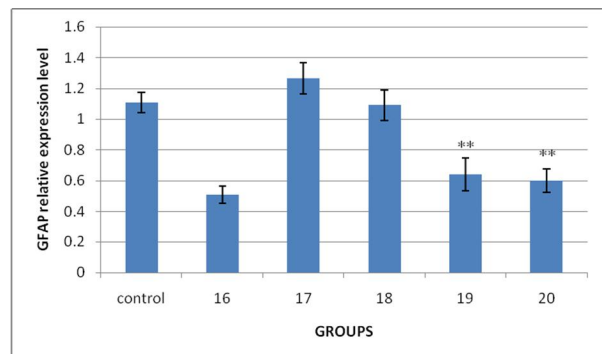


Fig 5: The effect of e-CSF on GFAP expression level. GFAP expression level significantly decreased in cultures exposed to E16, E19 and E20 CSF compared with control group. Data are means±S.E.M. (\*\*p<0.01& \*\*\*p<0.001).

In conclusion we have described evidences that suggest e-CSF induce morphological change in cultured astrocytes. However, further experiments are needed to understand mechanisms for the induction of these morphological changes.

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