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Dialogue Between Competence and Progression Growth Factors and Astroglial-Conditioned Media on Nucleic Acid and Erk1/2 Expression During Astroglial Cell Proliferation and Differentiation in Culture

Barbagallo A, Barbagallo F, Rapisarda G, Spampinato MR, Carota G, Distefano A, Conti G and Avola R*

Department of Biometer, Laboratory of Biochemistry, Via S. Sofia 97, 95123, University of Catania, Catania, Italy

ABSTRACT

Growth factors are classified in competence growth factors and progessing growth factors, as reported in a papaer by Stiles and Antonisse, published on 1970.

The competence growth factors, including basic fibroblast growth factors (bFGF) and PDGF, are protein that switch from G0 phase to G1 phase of the cell cycle.

The progression growth factors, including EGF, IGF-1 and insulin are proteins that, during S phase of the cell cycle, stimulate DNA biosynthesis by activating the enzymes DNA polymerase, thymidine kinase and timidilate synthetase.

The aim of this present investigation is to study the cross-talk between exogenous growth factors and endogenous growth factors released from astroglial-conditioned media on DNA labeling and ERK ½ expression in astrocytes in an in vitro model. To better clarify mechanism of astroglial cell proliferation / differentiation in culture, incorporation of [methyl-3H]-thymidine in astroglial cell cultures was investigated. Astrocytes cultures were pre-treated with epidermal growth factor (EGF), insulin (INS), insulin-like growth factor-I (IGF- I), and basic fibroblast growth factor (bFGF) and subsequently with astroglial conditioned media (ACM). In particular, the incorporation of [methyl-3H]-thymidine into DNA showed a significant increase in ACM from 15 days in vitro (DIV) cultures in 30 DIV astrocytes after12 h pre-treatment with growth factors. The results of enhancement in DNA labeling after pre- treatment with EGF or INS in 30 DIV astrocytes cultures and subsequent addition of ACM from 15 DIV cultures, indicate that the involvement may depend on extra cellular signal-regulated kinase (ERK1) activation.

In addition, the present study seeks to elucidate the interactions between the competence growth factor bFGF and/or estrogen 17b-estradiol and the progression growth factor EGF, IGF-1 and insulin on GFAP and vimentin expression in astroglial cultures under different experimental conditions. Pretreatment for 24hr with bFGF subsequent exposure for 36hr estradiol and EGF, IGF-1 or INS was markedly increased, when the cultures where treated with two or three growth factors. Our data demonstrate estradiol-growth factor cross-talk during astroglial cell proliferation and differentiation in culture.

In summary, the environment created by astroglial cultures can regulate their own proliferation and differentiation, through the release of soluble mediators finally acting on their genomic program. This may be relevant in vivo, when scheduled events related to brain development are regulated by astrocyte-derived growth factors controlling neuronal and glial architecture from the postnatal period until the adulthood.

***Corresponding author**

Avola R, Department of Biometer, Laboratory of Biochemistry, Via S. Sofia 97, 95123, University of Catania, Catania, Italy.

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Introduction

Growth factors are classified in competence growth factors and progessing growth factors, as reported in a paper by Stiles and Antonisse, published on 1970.

The competence growth factors, including basic fibroblast growth factors (bFGF) and PDGF, are protein that switch from G0 phase to G1 phase of the cell cycle.

The progression growth factors, including EGF, IGF-1 and insulin are proteins that, during S phase of the cell cycle, stimulate DNA biosynthesis by activating the enzymes DNA polymerase, thymidine kinase and timidilate synthetase. Growth factors are crucial molecules in the neuron-glia dialogue and may exert mitogenic and trophic effects during nervous system morphogenesis and development. Extra cellular medium enriched by secreted trophic factors during astrocyte proliferation and maturation in culture stimulates cellular growth and differentiation through an autocrine or paracrine loop. Astroglial-conditioned media (ACM) influence the development and differentiation of nerve cells cultures and may regulate biochemical, functional and morphological events correlated to neuron-glia cross-talk [1-3]. ACM collected from epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I)-treated astrocytes are enriched of plasminogen activator (PA) [4,5]. The competence factor (bFGF) as well as EGF, insulin (INS), IGF-I, and bFGF are released by astrocytes. INS and IGF-I are important mitogenic and trophic factors for neural cell line [6,7]. EGF enhanced development and maturation of astrocytes cultures and acts synergically with IGF-I [8-16].

The extra cellular signal-regulated kinase (ERK) cascade induced some immediate early genes (c-fos), (c-myc) (c-jun) controlling cell cycle progression [17,18].

Several previous studies have shown that astrocytes play active roles in the modulation of neuronal activity and synaptic neurotransmission [19]. Moreover, astrocyte activity may be modulated by neurosteroids and growth factors.

Glial fibrillary acidic protein (GFAP) is widely recognized to be an astrocyte differentiation marker, constituting the major intermediate filament (IF) protein of mature astrocytes [20-23].

Growth Factor, such as, EGF, IGF-I, and INS, act as mitogenic peptides and participate in neuron–glia cross-talk, stimulating cultured nerve cell proliferation and differentiation.

In addition, it is well known that estrogens and growth factors, such as EGF, act as mitogens, promoting cellular proliferation.

The aim of the present investigation concerns cross-talk between exogenous growth factors and endogenous growth factors released from astroglial-conditioned media (23) on DNA and RNA labeling and ERK1/2 expression in astrocytes in an in vitro model [24,25].

The present paper has observed the effect of ACM collected from 15, 30, 60 or 90 days in vitro (DIV) on astrocytes cultures during the development process (15 or 30 DIV) pre-treated with growth factors (EGF, IGF-I or INS). The present research was particularly assessed up and down modulation by exogenous growth factors during the dialogue with endogenous growth factors, released in ACM during proliferation and differentiation process in culture.

On the other hand, the present study is devoted to eiucidating the interactions between the "competence" growth factor bFGF and/or 17b estradiol (E2) and the "progression" growth factors (EGF, IGF-I, INS) on GFAP and vimentine expression and on proliferation and differentiation activity of primary astroglial cell cultures under different experimental conditions.

Experimental Procedures

Astroglial Cell Cultures

Primary astroglial cultures were obtained from cerebral hemispheres of newborn rats as previously described (10,14,15). Cells were cultured in Petri's plastic dishes (Falcon, 35 or 60 or 100 micrometers in diameter) at a density of 0.5-1 x 10^2 and were incubated at 37 degree in a 5% CO_2- humidified atmospheric air till they reaced confluence.

Approximately 96% cultured cells displayed GFAP immunoreactivity confirming their astrocyte nature.

Astroglial Conditioned Media

ACM used were collected from previous experiments. Material included 15, 30, 60 or 90 DIV 24 h serum deprived astrocyte cultures stored at –80°C until used.

Growth factors necessary for culture pre-treatment were added at the following concentrations: bFGF, 5 ng/ml; EGF, 10 ng/ml; IGF-I, 10 ng/ml; INS, 10µg/ml.

DNA Labeling Assay

[methyl-3H] thymidine (2µCi/ml culture medium) incorporation into DNA was assayed from growth factor-treated and untreated (control) astroglial cell cultures.

Astroglial cells were extracted with 1N perchloric acid for 30 minutes at 4°C. Acid insoluble material was washed three times with 0.5 N perchloric acid, once with ethanol and solubilized in 0.3 N NaOH at 37°C for 30 minutes.

Nucleic acid were then extracted as previously described (16) and aliquots were taken for radioactivity measurement. Radioactivity was expressed as dpm/mg of proteins.

Proteins were determined by the method of Lowry and co-workers (26) using bovine serum albumine as standard.

Erk1 Immunoblotting

After growth factors treatment, cells were rinsed two times with ice-cold phospate-buffered saline (PBS) with addition of orthovanadates to inhibit phosphatases.

Cells were then solubilized in a buffer containig 2% SDS 10% glicerol 50mM dithiotreitol and 0.1% bromophenol blue. Blots were washed three time in PBS-T and immunoprecipitate was visualized on a film by an ECL kit. The product of phospho-ERK1 immune reaction was quantified by computeried densitiometry (Scion Image program; Scion image corporation, USA).

Statistics

Values in the text were expressed as the means +/- standard error (SEM) of data obtained from five different dishes. The results of experiments of both DNA labeling were analyzed statistically by analysis of variance (ANOVA) followed by Duncan's multiple range test. Statistical significance was expressed at P values of <0.05, <0.01 and <0.001.

Results

Results INS

PANEL A (15 DIV)

UNTREATED CONTROL 4.110	INS PRETREATED CONTROL 5.110 ● DPM / mg PROTEINS	ACM 30 DIV 1.605 ■ DPM / mg PROTEINS	ACM 60 DIV 1.512 ■ DPM/ mg PROTEINS	ACM 90 DIV 1.197 ■ DPM/ mg PROTEINS

PANEL A (30 DIV)

UNTREATED CONTROL 905 DPM/ mg PROTEINS	INS PRETREATED CONTROL **1.220●** DPM /mg PROTEINS	ACM 15 DIV **15.045 ▲** DPM/mg PROTEINS	ACM 30 DIV **980 ●** DPM/mg PROTEINS	ACM 60 DIV **891●** DPM/mg PROTEINS	ACM 90 DIV **790●** DPM/mg PROTEINS

Addition for 24 h of ACM obtained at 30, 60 or 90 DIV significantly reduced DNA labeling in 15 or 30 DIV astrocytes pre-treated for 12 h with INS (Figures. A 15 div and A 30 div). A slight but significant increase of DNA labeling was found in INS-pretreated cultures at 15 or 30 DIV compared to control cultures (Figures. A 15 div and A 30 div).

A 12 h INS pretreatment remarkably increased DNA labeling. Hence, the greatest mitogenic effect was induced by INS pretreatment in 30 DIV cultures, treated for 24 h with ACM collected from 15 DIV developing cultures (A 15 div).

Addition of ACM to 15 DIV cultures from 30 or 60 or 90 DIV after 12 h pre-treatment with INS (Figures. A 15 div or A 30 div) markedly inhibited DNA labeling.

Results IGF-1
PANEL B (15 DIV)

UNTREATED CONTROL 4.150	IGF-1 PRETREATED CONTROL 4.190 DPM / mg PROTEINS	ACM 30 DIV 1.590 ▲ DPM / mg PROTEINS	ACM 60 DIV 1.625 ■ DPM/ mg PROTEINS	ACM 90 DIV 1495 ▲ DPM/ mg PROTEINS

PANEL B (30 DIV)

UNTREATED CONTROL **1.130** DPM/ mg PROTEINS	IGF-1 PRETREATED CONTROL **1.192** DPM /mg PROTEINS	ACM 15 DIV **5.030** ▲ DPM/mg PROTEINS	ACM 30 DIV **11000** DPM/mg PROTEINS	ACM 60 DIV **899** DPM/mg PROTEINS	ACM 90 DIV **585** ▲ DPM/mg PROTEINS

Addition for 24 h of ACM obtained at 30, 60 or 90 DIV significantly reduced DNA labeling in 15 or 30 DIV astrocytes pre-treated for 12 h with IGF- I (Figures. B 15 div and B 30 div). No differences were noticeable between control and IGF I-treated cultures at 15 (B 15 div) or 30 (B 30 div). Addition of ACM obtained at 15 DIV induced a marked stimulation of DNA labeling in 12 h Insulin-like growth factor I pretreated 30 DIV cultures (B 30 div). This effect was more pronounced after IGF-I Treatment. Addition of ACM to 15 DIV cultures from 30 or 60 or 90 DIV after 12 h pretreatment with IGF-I (Figure. B 15 div) markedly inhibited DNA labeling. Addiction of 90 div ACM to 30 div IGF1 pretreatment cultures significant decreased DNA labeling (B 30 div).

Results EGF
PANEL C (15 DIV)

UNTREATED CONTROL **4.190** DPM / mg PROTEINS	EGF-PRETREATED CONTROL **5.207** DPM / mg PROTEINS	ACM 30 DIV **2.025** DPM / mg PROTEINS	ACM 60 DIV **1.990** DPM/ mg PROTEINS	ACM 90 DIV **1960** DPM/ mg PROTEINS

PANEL C (30 DIV)

UNTREATED CONTROL **1.040** DPM/ mg PROTEINS	EGF PRETREATED CONTROL **1.800** DPM /mg PROTEINS	ACM 15 DIV **4.307** ▲ DPM/mg PROTEINS	ACM 30 DIV **880** ● DPM/mg PROTEINS	ACM 60 DIV **905** ● DPM/mg PROTEINS	ACM 90 DIV **635** ● DPM/mg PROTEINS

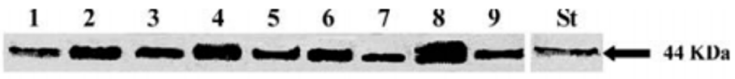
Addition for 24 h of ACM obtained at 30, 60 or 90 DIV significantly reduced DNA labeling in 15 or 30 DIV astrocytes pre-treated for 12 h with EGF (Figures. C (15 div) and C (30div).

A slight but significant increase of DNA labeling was found in EGF- pretreated cultures at 15 or 30 DIV compared to control cultures (Figures. C (15 div) and C (30div).

Addition of ACM obtained at 15 DIV induced a marked stimulation of DNA labeling in 12 h epidermal growth factor-pretreated 30 DIV cultures (C). This effect was more pronounced after EGF treatment.

Addition of ACM to 15 DIV cultures from 30 or 60 or 90 DIV after 12 h pretreatment with EGF (Figure. C 15 div) markedly inhibited DNA labeling.

Results Erk1 30 Div Cultures (Figure D)



1 21 UDO	2 45 UDO ■	3 31 UDO	4 51 UDO ■	5 27 UDO	6 41 UDO ■	7 25 UDO	8 68 UDO ▲	9 26 UDO

(1) Control; (2) EGF treated astrocyte cultures; (3) ACM 30 DIV in EGF pretreated cultures; (4) bFGF treated astrocyte cultures; (5) ACM 30 DIV in bFGF pretreated cultures; (6) IGF-I treated astrocyte cultures; (7): ACM 30 DIV in IGF-I pretreated cultures; (8) INS treated astrocyte cultures; (9) ACM 30 DIV in INS pretreated cultures; St: Standard.

Western blot analysis suggests that this sharp stimulating effect depends on activation of the Mitogen activated protein kinase member ERK1 (Figure .D). ERK-1 expression was increased in EGF or bFGF or IGF-I or INS treated astrocyte cultures at 30 DIV. No significant changes were observed when cultures treated with ACM 30 DIV were pre-treated with EGF or bFGF or IGF-I or INS (Figure.D), whereas a significant decrease in ERK-1 expression was found after addition of ACM 30 DIV in growth factor-pretreated cultures at 15 DIV (Figure. D).

Western Blot Analysis for Gfap Expression in Astrocyte Cultures at 24 Div
Effect of pretreatment with estradiol alone or along with two or three growth factors on GFAP expression.

GFAP expression in astrocyte cultures at 24 DIV was markedly increased when the cultures were treated with the neurotrophic fattors,especially treatment with EGF+bFGF, EGF+IGF-I, EGF+lNS,or bFGF+INS and also with EGF+bFGF+IGF-I or EGF+bFGF+INS compared with untreated controls and also with E2 36 h treatment.

1 16.000.000 Arbitrary Units	2 18.000.000 Arbitrary Units	3 30.000.000 Arbitrary Units ●	4 20.000.000 Arbitrary Units ●	5 33.000.000 Arbitrary Units ●	6 27.000.000 Arbitrary Units ●	7 38.000.000 Arbitrary Units ▲	8 45.000.000 Arbitrary Units ▲	9 48.000.000 Arbitrary Units ▲

1. Control;
2. E2 for 36 hr;
3. E2 for 36 hr+EGF+bFGF (Iast 24 hr);
4. E2for 36 hr+EGF+IGF-I (Iast 24 hr);
5. E2 for 36 hr+EGF+INS (Iast 24 hr);
6. E2 for 36 hr+bFGF+IGF-I (Iast 24 hr);
7. E2 for 36 hr+bFGF+INS (Iast 24 hr);
8. E2 for 36 hr+EGF+bFGF+IGF-I (Iast 24 hr);
9. E2 for 36 hr+EGF+bFGF+INS (Iast 24 hr).

Western Blot Analysis for Vimentin Expression in Astrocyte Cultures At 24 Div.
Effect of pretreatment with estradiol alone or along with two or three growth factors on Vimentine expression.

GFAP expression in astrocyte cultures at 24 DIV was markedly increased when the cultures were treated with the neurotrophic fattors,especially treatment with EGF+bFGF, EGF+IGF-I, EGF+lNS,or bFGF+INS and also with EGF+bFGF+IGF-I or EGF+bFGF+INS compared with untreated controls and also with E2 36 h treatment.

1 $2.2x10^{-7}$ Arbitrary Units	2 $2.3x10^{-7}$ Arbitrary Units ●	3 $4.0x10^{-7}$ Arbitrary Units ●	4 $3.8x10^{-7}$ Arbitrary Units ●	5 $3.9x10^{-7}$ Arbitrary Units ●	6 $3.9x10^{-7}$ Arbitrary Units ●	7 $4.3x10^{-7}$ Arbitrary Units ▲	8 $4.5x10^{-7}$ Arbitrary Units ▲	9 $4.7x10^{-7}$ Arbitrary Units ▲

Discussion
The competence growth factors, (bFGF) and platel derived growth factor, are protein that switch from G0 phase to G1 phase of the cell cycle.

The progression growth factors, including EGF, IGF-1 and insulin are proteins that, during S phase of the cell cycle, stimulate DNA biosynthesis by activating the enzymes DNA polymerase, thymidine kinase and timidilate synthetase.

ACM treatment of 30 DIV astrocytes pre-treated in the same way had no effect compared either versus untreated controls or versus 12 h growth factor-pre-treated cultures. ACM obtained from 30 or 60 or 90 DIV contain one or more soluble factors capable of suppressing DNA synthesis in early developing astrocytes (15 DIV). In differentiated astrocytes at 30 DIV, DNA labeling displayed less sensitivity to the effects exerted by ACM (Astrostatine). This is not true for ACM collected form 15 DIV astrocytes that stimulate DNA synthesis of 30 DIV astrocyte cultures.

The results suggest: a regulatory flow of trophic and mitogenic factors promoting cell interactions in the astroglial compartment. A particular interest comes from the demonstration of relevant effects elicited by INS or EGF in 30 DIV cultures, when added for 12 h before treatment for 24 h with ACM from 15 DIV cultures.

The treatment with ACM from 30 or 60 or 90 DIV cultures, following a 12 h growth factor-pretreatment period, is associated with a reduction of DNA labeling. An unexpected finding was the effect of ACM on 30 DIV cultures after 12 h bFGF pretreatment.

bFGF pre-treatment did not enhance DNA labeling after addition of ACM collected from 15 DIV cultures, whereas this effect is induced by EGF, IGF-I or INS pre-treatment. This suggests that bFGF behaves like a differentiation-promoting agent.

bFGF, INS and EGF induce astroglial cell differentiation depending of the culture stage. Between birth and the stage of 25–30 DIV, astrocytes probably synthesize and release in the medium factors having mainly a proliferative effect, while after the 13th day of culture they progressively release growth arresting and differentiation-promoting molecules (Astrostatine).

Mitogenic growth factors themselves regulate the production of growth-arresting mediators and therefore they may control astroglial cell differentiation once the genomic clock established the appearance of the differentiated phenotype.

Our data collectively show that ACM derived from 15 DIV young astrocyte cultures stimulate proliferation of 15 and 30 DIV astrocyte cultures pre-treated for 12 h with growth factors, while growth of astrocytes is blocked after treatment with ACM collected from 30 or 60 or 90 DIV cultures in the same conditions.

Our findings demonstrate a differential up- or down-regulation of DNA labeling in astrocyte cultures pre-treated with exogenously added growth factors or treated with endogenous trophic molecules released in the conditioned media.

Finally, our findings on GFAP and Vimentine expression suggest an intertwined dialog between these two classes of neuroactive molecules and confirm the complex role played by estradiol and both competence and progression growth factors in regulating specific protein expressions during astroglial cell proliferation and differentiation. This dialog could have an important involvement in future therapeutic strategies correlated with neurological and neurodegenerative disorders.

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