17β-Estradiol enhances neuronal differentiation of mouse embryonic stem cells

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Abstract Existing protocols show a variety in the percentage of neurons that can be generated from mouse embryonic stem (ES) cells. In the current study, we compared effects of various differentiating conditions, including gelatin and poly-L-ornithine/fibronectin coatings, and NGF and 17-estradiol treatments on the total yield of neurons, as well as, neurite growth and branching. Here, we show that combination of fibronectin coating with 17-estradiol increased number of generated neurons over 50%. Poly-L-ornithine/fibronectin increased the percent of neurons in all cultures, suggesting its direct influence on neurogenesis. Addition of 17-estradiol reduced mean neurite length in culture, but significantly increased branching. Our results indicate a substrate-dependent regulation of estrogen-induced ES cells differentiation into neuronal cells.

Keywords: Poly-L-ornithine/fibronectin; 17β-Estradiol; Differentiation; Embryonic stem cell; Neuron; Neurite; Branching

1. Introduction

Embryonic stem (ES) cells derived from a blastocyst differentiate into a variety of cell types. Experiments have shown that ES cells can be maintained in culture over a year (more than 250 passages) while retaining their phenotype and ability to differentiate [1]. Because ES cells may be easily cultured, propagated and genetically manipulated, they may prove to be a good source of specialized cells for transplantation applications. Targeted differentiation of stem cells into specific neuronal and glial phenotypes holds promise to develop into a powerful approach for treatment of neurological disorders and injuries. There are reports showing that ES cells can be successfully used for treatment of animal models of spinal cord injury [2,3], and Parkinson's disease [4–6]. However, generation of specific neuronal populations in sufficient quantities for transplantation represent a significant practical problem. Several protocols have been successfully used to generate neurons from stem cells. These include treatments with retinoic acid [7], ascorbic acid [5,6], BMP-2 [8], and Shh proteins [9]. However, the reported percentile of cells expressing neuronal markers is usually low and does not exceed 30%. Different conditions that may affect ES cell differentiation include: cell culture media, cell flask coating and growth factors. In the current study, we compared the effects of various differentiating conditions, including gelatin and fibronectin coatings, and NGF and 17β-estradiol treatments on a total yield of neurons, as well as, on neurite growth and branching. We found that poly-L-ornithine/fibronectin induces neuronal phenotype. A combination of poly-L-ornithine/fibronectin with 17β-estradiol reduced mean neurite length in culture, but significantly increased branching. These results demonstrate a substrate-dependent regulation of estrogen-induced neuronal differentiation of ES cells.

2. Materials and methods

2.1. Cell culture and differentiation

Mouse ES cells (WW6, American Type Culture Collection, Manassas, VA) were routinely passaged every two days in gelatinized 25 cm flasks at a density $\sim 1 \times 10^6$ cells in DMEM with high glucose (Stem Cells Technologies) in the presence of ESGRO (recombinant mouse LIF) 1000 U/ml (Chemicon International, Temecula, CA) and 1× penicillin/streptomycin (Gibco, Invitrogen Corporation, Carlsbad, CA). For induction of differentiation a modified 4–/4+ protocol [7] was employed. ES cells were first harvested from confluent flask. Cells were washed with Ca^{2+}/Mg^{2+}-free D-PBS, trypsinized (2 ml of 0.25% trypsin/EDTA) for 2–3 min, resuspended in 5 ml of IMEM-10% differentiation fetal bovine serum (FBS) (Gibco) and centrifuged (1200 rpm, 8 min). Approximately 1/4 ($\sim 3 \times 10^6$ cells) of ES cells from one nearly confluent (85–90%) 25 cm flask was resuspended in 10 ml of differentiation medium (DM) (IMDM, 15% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, penicillin 100 U/ml, streptomycin 100 μg/ml) and cultured in 100 mm non-adhesive Petri dishes to allow cells to aggregate and form embryoid bodies (EBs). Medium was replaced every two days. After four days EBs were gently transferred to a 15 ml tube, and allowed to stand until the EBs settled to the bottom (about 10 min). Medium was replaced and EBs were transferred back into the original dish by pipetting. At day 5 all *trans*-retinoic acid (Sigma, St. Louis, MO) (1×10^{-6} M) was added and the cells were cultured for another four days. After four days with retinoic acid, EBs were trypsinized and gently resuspended. Cells were plated on gelatin or poly-L-ornithine/fibronectin coated glass cover slips (~275 000 cells per cover slip) in DM medium. The next day the medium was changed and NGF(100 ng/ml, Sigma) or 17β-estradiol (1×10^{-8} M, Sigma) were added. The medium was changed and the treatment was repeated on the following day. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) and processed for immunocytochemistry.

2.2. Immunocytochemistry

Immunocytochemical staining was performed according to our laboratory protocol, as described previously [10]. Cells were first washed for 5 min in PBST (phosphate-buffered saline with 0.3% Triton X-100) and then incubated for 10 min in ice-cold 80% methanol containing 0.3% hydrogen peroxide to abolish endogenous peroxidase activity. Cells were then washed three times in PBST for 5 min, then

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incubated in avidin and biotin 15 min consequently (Avidin/Biotin Blocking Kit, Vector Laborastories, Burlingame, CA), rinsed with PBS, followed by a 1 h incubation with blocking solution, 10% normal goat serum in PBST. Cells were incubated with primary rabbit anti-MAP-2 polyclonal antibodies (greatest immunoreactivity with neuronal MAP-2A and MAP-2B) (Chemicon) at a 1:2000 dilution overnight at 4 °C. Subsequently, they were washed three times for 10 min with PBST and incubated with secondary biotinylated antibodies for 1 h. Cells were washed again three times in PBST for 10 min and incubated for 2 h in Elite ABC reagent (avidin-biotinylated horseradish peroxidase complex, Vector Laboratories). Cells antigens were visualized with a DAB Substrate Kit for Peroxidase (Vector Laboratories). Staining without primary or secondary antibodies served as negative controls (results not shown). After staining, coverslips were air dried, incubated in histoclear two times for 15 min and permanently mounted with Permount (Fisher Scientific). Images were taken using a Nikon Eclipse TS100 inverted microscope with attached Kodak DC290 digital camera and an Olympus IMT-2 inverted microscope with attached Spot digital camera.

2.3. Quantification of neurons, and measurement of neurite length and neurite branching

Quantification of neuron counts and measurement of neurite length and branching was performed according to protocol described elsewhere [11]. To visualize neuronal morphology, cells were stained with neuron-specific anti-MAP-2 polyclonal antibodies (Chemicon). Images of the cells were taken at 40× magnification using Spot digital camera and Spot Software v.3.5.8 (Diagnostic Instruments, Sterling Heights, MI). 10–30 individual images were taken from each cover glass. The number of neurons and the total number of cells were counted using NIH ImageJ software (NIH). Neurite length was measured only in neurons which were completely distinguishable from neighboring cells. ImageJ software was used for tracing neurites, as well as, for calculation of neurite length. The number of neurite branches was counted manually after the entire neuron was traced. Processes initiated from the cell body were scored if they extended greater than 1 cell body in length from the neurite, and secondary and higher order branches of the neurite were scored after a branch point. All experiments were repeated three times.

2.4. Statistical analysis

Statistical analysis was performed with one-way ANOVA Newman–Keuls Multiple Comparison Test using Prism (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA).

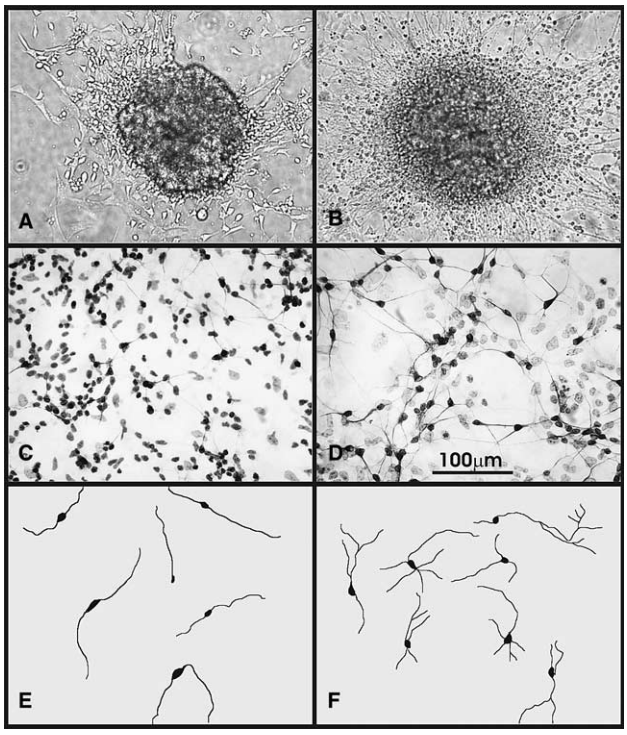

Fig. 1. ES cell differentiation. Upper panel: Phase contrast of ES cell aggregates plated on gelatin or poly-L-ornithine/fibronectin coated glass cover slips for 24 h. (A) Irregular shape of ES cells attached to gelatin substrate. (B) Many round-shaped cells with growing neurites are visible on poly-L-ornithine/fibronectin coated glass. Middle panel: MAP-2 positive cells on gelatin glass cover slips visualized by immunoperoxidase staining. (C) Few short neurites are visible in culture without any treatment. (D) Increase in neurite growth in the presence of 17β-estradiol added for 48 h. Lower panel: Tracings of neurons in ImageJ (NIH Image) program. (E) Bipolar cells can be observed on poly-L-ornithine/fibronectin coated glass cover slips without any treatment. (F) Increase in neurite branching after 17β-estradiol was added for 48 h.

3. Results

We examined live cultures with inverted microscope 24 h after plating ES cells onto gelatin or poly-L-ornithine/fibronectin coated glass cover slips. The analysis revealed a remarkable difference between ES cells growing on gelatin and on poly-L-ornithine/fibronectin coated cover slips. On gelatin cover slips, aggregates of ES cells had an irregular or spindle-like shape with fewer processes (Fig. 1A). On poly-L-ornithine/fibronectin coated cover slips the cells shape was predominantly round and aggregates showed striking neurite outgrowth (Fig. 1B). These data showed that poly-L-ornithine/fibronectin substrate encourages both neuronal differentiation and neurite outgrowth of ES cells.

The cultures were then treated with either NGF (100 ng/ml) or 17β-estradiol (1×10^{-8} M) for 48 h. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) and processed for immunocytochemistry for MAP-2, a neuron-specific marker. Immunohistochemical staining revealed a significant increase in the number of MAP-2-immunopositive cells on poly-L-ornithine/fibronectin versus gelatin coating (Fig. 2, upper panel). Interestingly, both NGF and 17β-estradiol treatments markedly increased number of neurons on poly-L-ornithine/fibronectin ($P < 0.001$), but not on gelatin. Statistical analysis with ANOVA of the number of neurons generated after treatment with NGF or 17β-estradiol on gelatin and poly-L-ornithine/fibronectin showed that the increase in number of neurons was possibly due to poly-L-ornithine/fibronectin coating rather than to treatments with NGF or 17β-estradiol.

Comparison of mean neurite lengths demonstrated the expected increase of neurite growth ($P < 0.001$) on poly-L-ornithine/fibronectin in compare to gelatin. Suprisingly, both NGF and 17β-estradiol increased mean neurite length on gelatin coating, but significantly reduced it on poly-L-ornithine/fibronectin (Fig. 2, middle panel). A small, but significant increase in neurite growth on gelatin in the presence of 17β-estradiol can be also observed in the microphotograph shown in Fig. 1D. What was interesting here is that poly-L-ornithine/fibronectin, which enhances neurite growth of different neural cell types [12,13], did not support neurite growth of differentiating ES cells in the presence of NGF or 17β-estradiol.

We further examined whether NGF and 17β-estradiol may effect neurite branching. Quantification of branching revealed no differences in the mean number of branches per cell on gelatin or poly-L-ornithine/fibronectin coatings (Fig. 2, lower panel). However, NGF treatment elicited a small non-significant

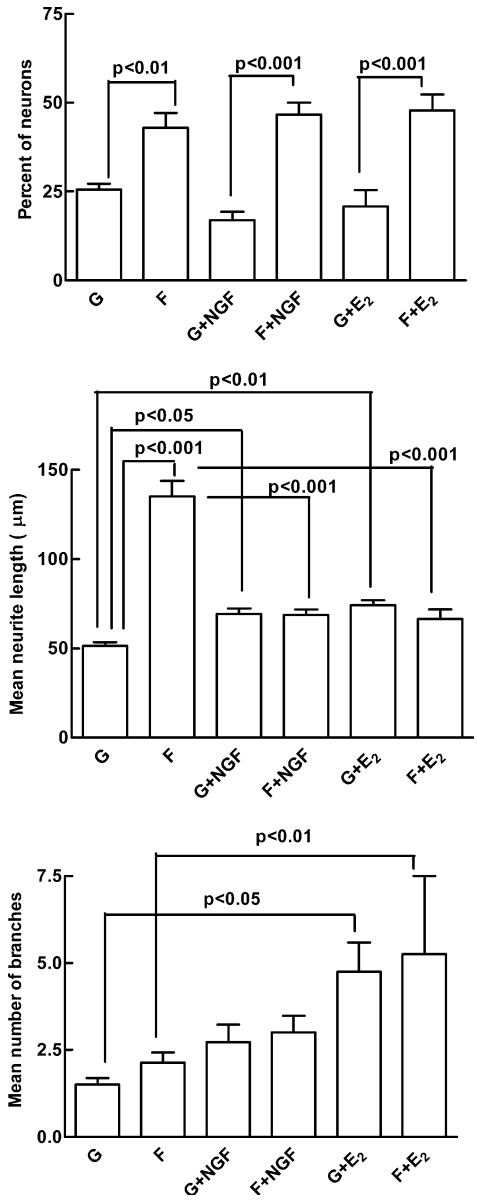

Fig. 2. Poly-L-ornithine/fibronectin increases percent of neurons and mean neurite length, while 17β-estradiol treatment induces branching. Upper panel: percent of neurons. Middle panel: mean neurite length (μm). Lower panel: mean number of branches. G, gelatin coating; F, poly-L-ornithine/fibronectin coating; G + NGF, gelatin coating plus NGF treatment; G + E2, gelatin coating plus 17β-estradiol; F + NGF, poly-L-ornithine/fibronectin coating plus NGF treatment; F + E2, poly-L-ornithine/fibronectin coating plus 17β-estradiol.

increase in neurite branching in each cell population on both gelatin and poly-L-ornithine/fibronectin. Treatment with 17β-estradiol produced a striking induction of neurite branching on both gelatin and poly-L-ornithine/fibronectin, which could be observed in ImageJ tracings shown in Fig. 1F. Statistical analysis by ANOVA of mean number of branches of 17β-estradiol treated cells on gelatin and poly-L-ornithine/fibronectin revealed that most of the increase in neurite branching was primarily due to 17β-estradiol treatment, rather than to substrate coating (Fig. 2, lower panel). Together with results on neurite length, these data indicated that the observed increase in estrogen-stimulated neurite outgrowth was not due to increase in neurite elongation, but instead to neurite branching.

4. Discussion

Our results illustrate an interesting interaction between trophic factors and extracellular matrix molecules in regulating differentiation of ES cells. Here, we showed that poly-L-ornithine/fibronectin coating alone can generate over 40% neurons. Fibronectin is a part of extracellular matrix in the central nervous system and is one of the most commonly used substrates for neuronal cell cultures providing adequate support for cell growth [12]. It has been recently shown that fibronectin may also act as a morphogen inducing neuronal differentiation [13]. In our unpublished observation we found that embryoid bodies treated for four days with fibronectin (10 μg/ml) added directly to the media generated neurons in a pattern similar to retinoic acid treatment. This indicates that fibronectin may be used as a powerful morphogen for ES cell neuronal differentiation.

The most striking observation was the interaction between poly-L-ornithine/fibronectin coating and trophic factors. NGF and 17β-estradiol produced small but significant increases in neurite length on gelatin, and a dramatic decrease on poly-L-ornithine/fibronectin. Therefore, the ability of fibronectin to stimulate neurite growth was inhibited in the presence of either NGF or 17β-estradiol. These results demonstrate a substrate-dependent regulation of NGF- and estrogen-induced neurite elongation. At the same time both NGF and, in particular, 17β-estradiol promoted neurite branching. 17β-estradiol significantly increased neurite branching on both gelatin and poly-L-ornithine/fibronectin coatings. NGF had a similar trend, however, it failed to reach statistical significance in our experiments. What was surprising here is that 17β-estradiol on poly-L-ornithine/fibronectin substrate significantly decreased mean neurite length while significantly inducing branching. This may indicate accelerated differentiation of neurons which have shorter but better branched out neurites. Based on our data, we hypothesize that 17β-estradiol accelerates both differentiation and maturation of neuronal cells.

It is well known that estrogen may affect cell growth, proliferation, and differentiation [14]. In the nervous system estrogen has a variety of beneficial effects including protection from apoptosis [15], accelerated peripheral nerve regeneration [16], and increased neurite outgrowth [17]. It has been recently reported that 17β-estradiol may increase the ratio of neurons to glia cells in neural stem cells, suggesting an influence on neurogenesis during embryonic development [18]. In our report, we provide evidence of 17β-estradiol differentiating action on ES cells. To the best of our knowledge this is the first observation of morphogen-like effect of 17β-estradiol on induction of neuronal phenotype in ES cell culture. Moreover, our results demonstrate a substrate-dependent regulation of 17β-estradiol induced ES cells differentiation. Combination of 17β-estradiol with poly-L-ornithine/fibronectin may provide a beneficial growing condition for neuronal induction of ES cells.

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