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Short communication

Induced neuronal differentiation of human embryonic stem cells

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Abstract

Human embryonic stem (ES) cells are pluripotent cells capable of forming differentiated embryoid bodies (EBs) in culture. We examined the ability of growth factors under controlled conditions to increase the number of human ES cell-derived neurons. Retinoic acid (RA) and nerve growth factor (βNGF) were found to be potent enhancers of neuronal differentiation, eliciting extensive outgrowth of processes and the expression of neuron-specific molecules. Our findings show that human ES cells have great potential to become an unlimited cell source for neurons in culture. These cells may then be used in transplantation therapies for neural pathologies.

Theme: Development and regeneration

Topic: Cell differentiation and migration

Keywords: Embryonic stem cell; Nerve growth factor; Retinoic acid; Neuronal differentiation; Transplantation therapy

Embryonic stem (ES) cells are undifferentiated pluripotent cells derived from the inner cell mass of blastocyst stage embryos. These unique cell lines have the potential to form virtually any cell type in the body and can be propagated in vitro indefinitely in an undifferentiated state [18]. Recently, ES cell cultures were established from human embryos [17,22]. These cells are capable of forming embryoid bodies (EBs) that contain cells from all three embryonic lineages [10]. Human ES cell differentiation can be modulated by the addition of growth factors [19]. In mice, it has been shown that when ES cells are allowed to aggregate to EBs, they can differentiate, among many other cell types, into mature neurons [2,23], capable of innervating adult brain and spinal cord [6,12,14]. Thus, the prospects of using in-vitro differentiated human ES cells to replace CNS neurons are most promising.

We have here characterized the neurogenic effects of retinoic acid (RA), β nerve growth factor (βNGF) and transforming growth factor β1 (TGFβ1) on human ES cells in-vitro. Differentiation was initially assayed by in-situ hybridization to neurofilament light chain (NF-L) RNA, which is expressed by both immature and fully differentiated neurons [4]. We found that βNGF and high concentration of RA increased the proportion of neuronal cells in the cultures from 21% in controls to 39% and 52%, respectively. A lower concentration of RA (10^{-7} M instead of 10^{-6} M) produced an effect similar to NGF, whereas no effect on neuronal differentiation was observed with TGFβ1 treatment (Fig. 1).

Based on these results, we further examined the effects of RA on the differentiation of human ES cells into neurons. Using immunostaining we could detect neurofilament heavy chain (NF-H) protein, (found only in mature neurons [4]) in sections of mature EBs (Fig. 2a). When the EBs were plated, these neurons formed filamentous 'networks' (Fig. 2b) of various complexities which were stained positive for NF-H (Fig. 2c). Treatment with RA and consequent plating caused an increase in the number of EBs containing NF-H positive neurons to 76%, compared to 54% in untreated EBs. Moreover, the RA-treated EBs showed dramatic shift towards more complex network

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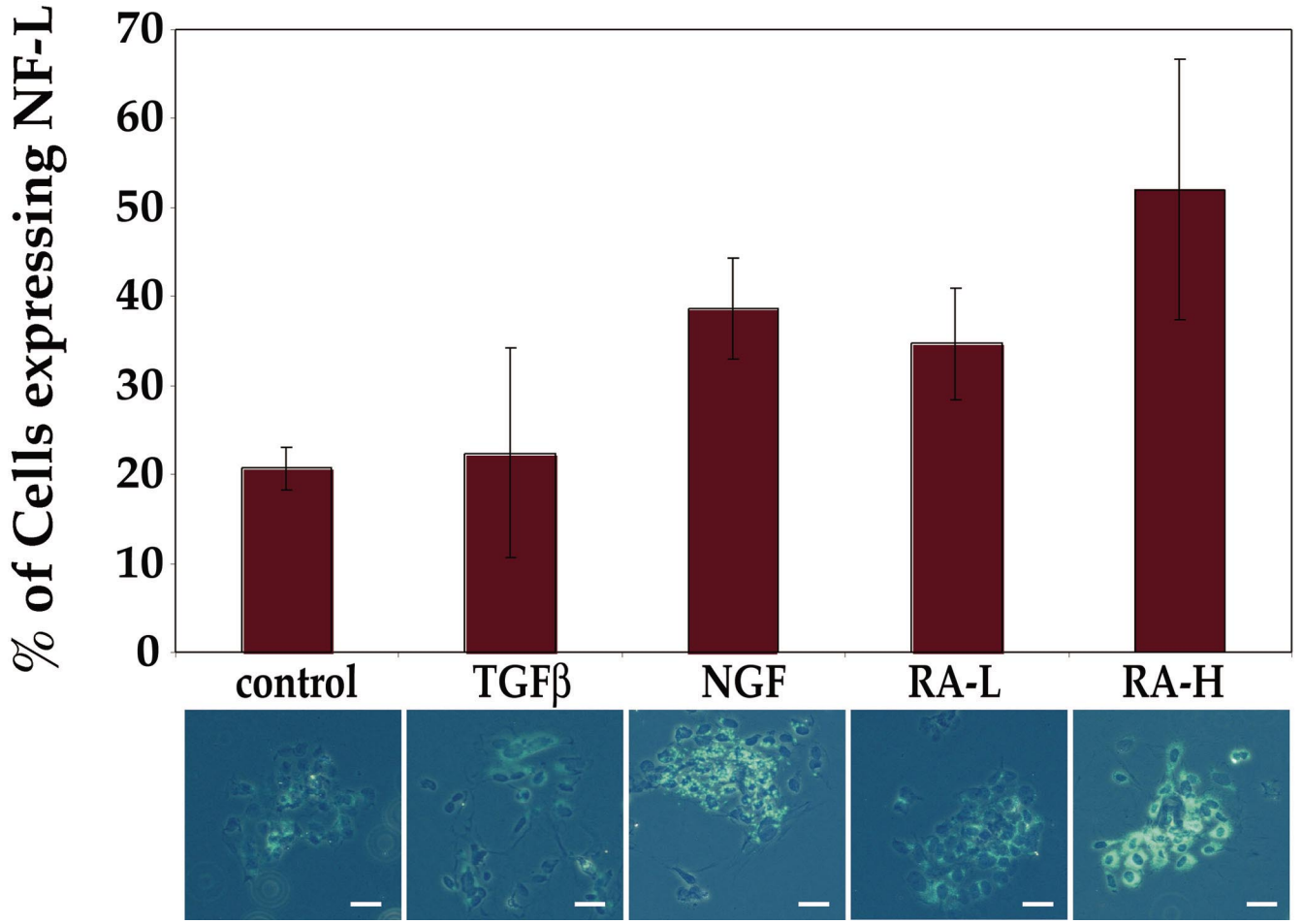

Fig. 1. Retinoic acid and βNGF induce expression of neurofilament light chain mRNA. In situ hybridization analysis of cells derived from dissociated EBs demonstrating expression of NF-L. Shown are RA- (low (10^{-7} M) or high (10^{-6} M) concentrations), TGFβ1, and βNGF-treated cultures. Scale bar=100 μM. Histograms show the percentage of cells expressing the NF-L RNA following each treatment. Assay of each condition was repeated three times (between 50 and 150 cells) and standard error bars are shown in the graph.

morphology (Fig. 2d). This shift may show an effect of the growth factor on the differentiation status of the cells, but may also be a by-product of the enhancement of neuronal cell numbers.

To characterize the nerve cells obtained from human ES cells, we assayed for expression of a dopamine receptor (DRD1) and two serotonin receptors (5HT2A and 5HT5A) in EBs treated with RA and in ES cells as a negative control (Fig. 3). These receptors were detected in EBs following RA treatment, but not in naive ES cells. Since dopamine and serotonin receptors were expressed, it was interesting to determine whether neurons in the cultures also synthesized their neurotransmitter ligands. Consistent with this possibility, we observed the expression of dopa decarboxylase (DDC), a key enzyme in the synthesis of both serotonin and dopamine [21] (Fig. 3).

We demonstrate here that both RA and βNGF enhance the number of neuronal cells that develop from human ES cells, as was observed for mouse ES cells when used at similar concentrations [1,20]. In addition we show that RA promotes the production of mature neurons and that these neurons express dopamine or serotonin receptors, and form complex plexuses of neuronal processes. The enrichment of neurons in culture by growth factors could have been through a process of cell selection (either by expansion or improved survival) or as a consequence of induced differentiation. Our production of neurally-differentiated ES cells provides new possibilities for obtaining large amounts of neurons in culture. These cultures should be investigated further in the future in order to demonstrate the expression of various receptors and developmentally regulated proteins specific to the nervous system. Moreover, once the functional capacity of the neural cells is demonstrated in-vitro and in-vivo, these cells may be useful for the replacement of neurons lost to degeneration or trauma in the CNS. The use of this unlimited cell source, alongside that of neuronal stem cells [7,11], may reduce the need for difficult-to-obtain fetal tissues, which to date are the primary sources of human cells available for transplantation therapy [16]. From experiments with both mouse and human ES cells, it is clear that though possible [3,15], it will be difficult to produce a homogenous culture

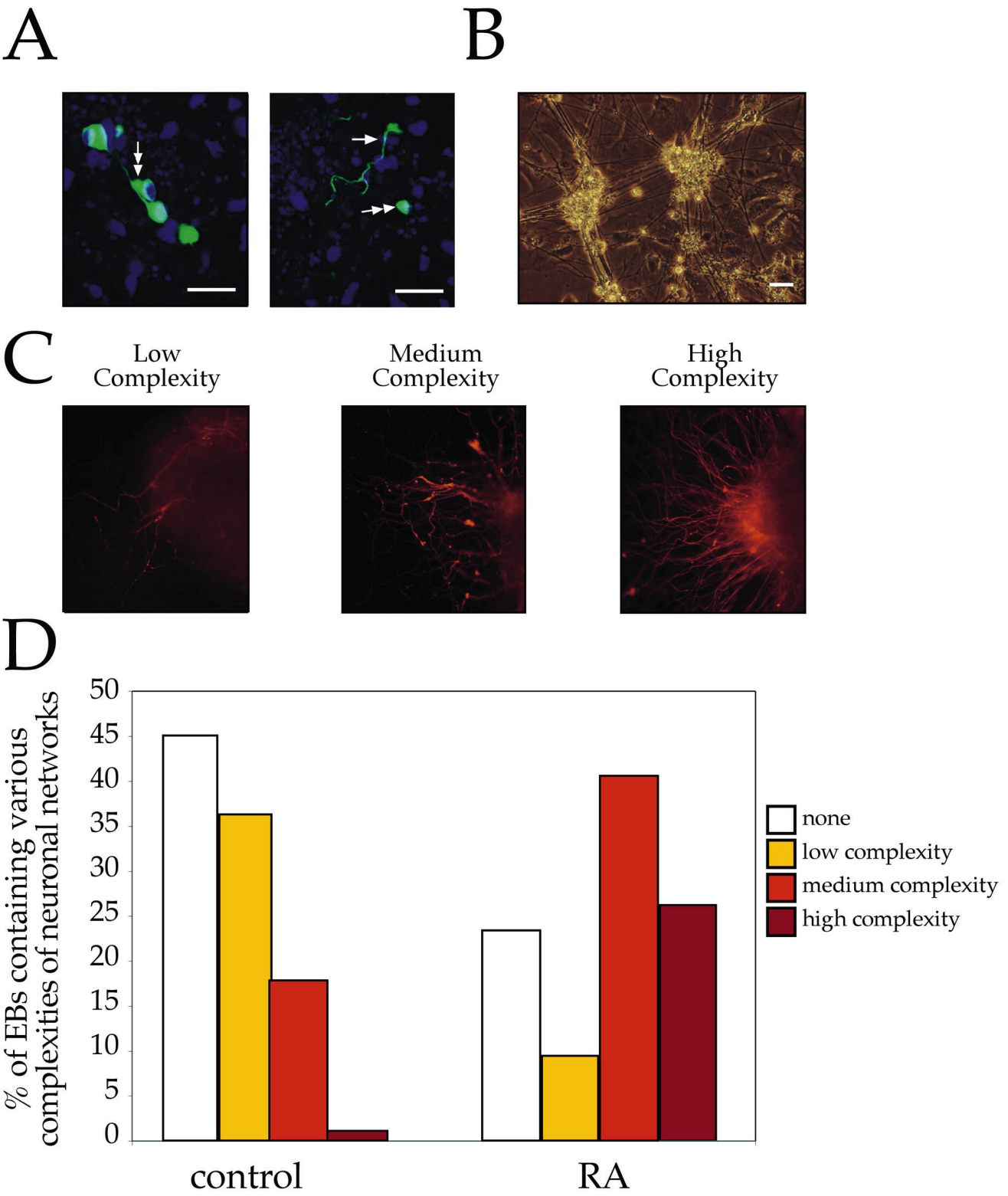

Fig. 2. Retinoic acid enhances formation of neuronal processes from human EBs. (A) Neuronal cell bodies (double arrows) and a complex proximal process (arrow) are seen in a section through an EB stained with anti-NF-H antibody. Hoecsht staining (blue) shows both intact and fragmented nuclei. (B) A plexus of neuronal-like processes formed from intact EBs plated on collagen. (C) Immunostaining for NF-H of 20-day-old EBs plated on collagen shows that plexuses of neuronal processes with varying degrees of complexity develop. (D) A histogram summarizing the effect of RA on the proportion of EBs displaying different degrees of network complexity. Each condition was assayed by counting all EBs in culture (between 20 and 40) and the experiment was repeated twice resulting in similar results. Scale bar=100 μM.

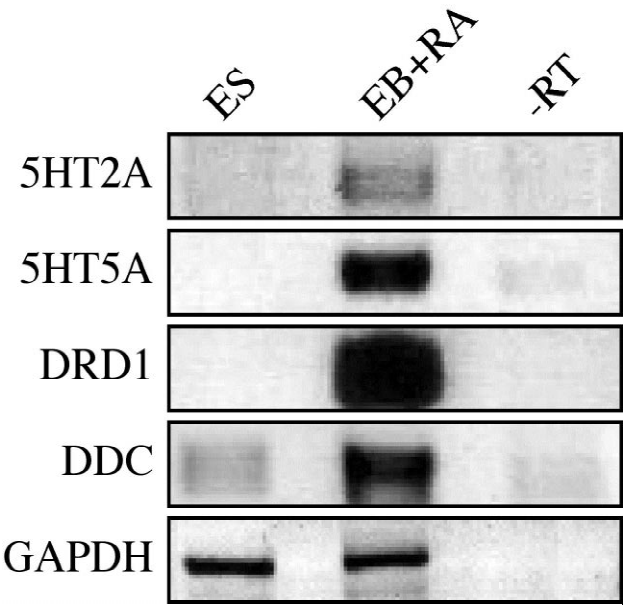

Fig. 3. Retinoic acid induces expression of neuron-specific genes. RT-PCR analysis of expression of dopamine receptor D1 (DRD1), serotonin receptor 2A and 5A (5HT2A and 5HT5A), dopa decarboxylase (DDC) and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human embryonic stem (ES) cells, and in 21-day-old EBs in the presence of RA (EB+RA). RNA samples that were not reverse transcribed were used as a negative control to ensure the absence of DNA contamination (−RT).

of neuronal cells without the use of lineage selection. This could be performed using genetically modified ES cell lines expressing antibiotic resistance [13] or marker genes [8] under cell specific promoters. The ability to genetically manipulate human ES cells [8] combined with directed neuronal differentiation may prove human ES cells as a valuable cell source for basic research and transplantation therapy.

Human ES cells (H9 clone [22]) were grown and EBs were generated as described [10]. Four days following initiation of aggregation, growth factors were administered: retinoic acid (Sigma): 10^{-7} M [2] or 10^{-6} M [1]; TGFβ1 (Sigma): 2 ng/ml [20]; βNGF (New Biotechnology, Israel): 100 ng/ml [23]. After 21 days, EBs were plated on 5 μg/cm^2 collagen-treated plates, either as whole EBs, or as single cells dissociated with trypsin/EDTA. The cultures were maintained for an additional week or 2 days, respectively.

EBs or their plated derivatives were fixed in 4% paraformaldehyde. In in-situ experiments, hybridization to a 50-mer 2′-O-methyl 5′-biotinylated cRNA of NF-L (CCTGCGTGCGGATGGACTTGAGGTCGTTGCTGATGGCGGCTACCTGGCTC) followed. The probes were then detected with streptavidin-conjugated alkaline phosphatase using a fluorogenic substrate [9]. For immunohistochemistry, EBs were either embedded in paraffin and sectioned at 10 μM, or stained directly on the plates. Mouse monoclonal anti-NF-H (a gift from Drs. Avihu Klar and Thomas Jessel) was used as a primary antibody, and was detected with Cy3-conjugated goat anti-mouse IgG.

Total RNA was extracted as described [5] and treated with RNAse free DNAseI. cDNA was synthesized from 1 μg total RNA using random hexamer as primer. cDNA samples were subjected to PCR amplification using the Clontech Advantaq+™ RT-PCR kit with a two-step cycle at 68°C. Primers for GAPDH, serotonin receptors 2A and 5A (5HT2A, 5HT5A respectively) and dopamine receptor D1 (DRD1) were purchased from Clontech. The primers for dopa decarboxylase (DDC) were: TCTGTGCCTCTTAACTGTCACTGTGG and ATCATCACAGTCTCCAGCTCTGTGC.

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