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In vitro differentiation of embryonic stem cells into glial cells and functional neurons

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SUMMARY

Mouse embryonic stem cells were induced to differentiate in culture with retinoic acid. Putative precursors of neurons and glial cells (nestin-positive cells) were clearly identified as early as three days after the onset of differentiation. At day 6, neuron-like cells could be clearly identified, either as isolated cells or as cellular networks. Some of these cells were positive for astrocyte- or oligodendrocyte-specific antigens (GFAP or O4 antigens, respectively). Other cells were positive for neuron-specific antigens (cytoskeleton proteins MAP2, MAP5 and NF200, as well as synaptophysin). Some neuronal-like cells were also positive for acetylcholinesterase activity or glutamic acid decarboxylase expression, indicating that ES cells could differentiate into GABAergic and possibly cholinergic neurons. Electrophysiological analyses performed in voltage clamp conditions showed that cell membranes contained voltage-dependent channels. Overshooting action potentials could be triggered by current injection. Taken together, these data provide evidence that embryonic stem cells can differentiate first into neuron-glia progenitors, and later into glial cells and functional neurons, in vitro. This technique provides an unique system to study early steps of neuronal differentiation in vitro.

Key words: mouse embryonic stem cell, neuronal differentiation, neuron, glial cell, neuronal precursor cell

INTRODUCTION

Mouse embryonic stem cells (ES cells) are continuously growing cells derived from the inner cell mass of the 3.5 day blastocysts (Evans and Kaufman, 1981; Martin, 1981). ES cells can be cultured in an undifferentiated state in vitro for extended periods of time. When reintroduced back into mouse blastocysts, they retain the ability to contribute to all cell lineages of the developing mouse, including the germ line (Bradley et al., 1984; Robertson, 1987). It has been shown that ES cells also retain the ability to generate differentiated progeny in vitro (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al., 1985). This implies that ES cells can both generate and respond in vitro to signals that normally regulate murine development. ES cells can form three-dimensional structures described as embryoid bodies, from which they differentiate spontaneously into a large variety of cell types (Robertson, 1987; Smith et al., 1988). Retinoic acid (RA), DMSO and methoxybenzamide are also potent differentiation inducers commonly used to trigger differentiation of ES cells more specifically into endoderm-like and fibroblast-like cells (Robertson, 1987; Smith, 1991). In the past few years, several in vitro models of ES cell differentiation have been described. One of the most well characterized is an in vitro model of hematopoietic differentiation (Schmitt et al., 1991; Wiles and Keller, 1991; Keller et al., 1993). Using this model, cytokine and receptor gene expression involved in hematopoietic differentiation have been studied (Schmitt et al., 1991; Keller et al., 1993). In vitro cardio-muscular differentiation of ES cells was also shown to closely recapitulate the early steps of muscle development in vivo (Wobus et al., 1988). These data have paved the way for a new experimental method to study lineage development and differentiation in vitro.

There has been a growing interest, over the past few years, in the characterization of cell lineages during neuronal differentiation of primary cultures (Raff and Miller, 1983; Price et al., 1991; Davis and Temple, 1994) as well as in the molecular mechanisms involved in cell type determination and differentiation (Anderson and Axel, 1986). An in vitro model that would recapitulate the whole differentiation pathway from uncommitted embryonic cells to functional neurons would be an efficient tool to study neuronal differentiation. Embryonal carcinoma (EC) cells have been shown to differentiate into neuronal cells in vitro (Lang et al., 1989; Imrik and Madarasz, 1991). However, EC cells are developmentally compromised embryonic stem cells that seldom contribute to all differentiated cell types and form tumors in chimaeric mice following their reintroduction into the blastocyst (Robertson and Bradley, 1986). Preliminary evidence that ES cells can differentiate into neuronal-like cells in vitro have previously been reported

(Wobus et al., 1988; Lee et al., 1994). However, the ability of ES cells to differentiate into the whole spectrum of functional neurons and glial cells that characterize the CNS is still lacking.

In this report, we provide evidence that ES cells can differentiate into glial cells (astrocytes and oligodendrocytes) as well as into functional neurons (at least GABAergic and possibly cholinergic neurons) in vitro, with well reproducible kinetics. Neuron-glia precursor cells were also identified, providing a unique experimental model to study early steps of neuronal differentiation in vitro. During preparation of this paper, Bain et al. (1995) showed also that ES cells can generate neurons in vitro.

MATERIALS AND METHODS

Cells and media

The ES cell line CGR8 (free of mycoplasma contamination), derived from 129 Sv mice, was maintained undifferentiated without feeder cells, in the presence of DIA/LIF (Smith et al., 1988). ES cells were cultured at 37°C in 7.5% CO_2 in Glasgow's modified Eagle's medium (GMEM) supplemented with 10% foetal calf serum, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids (Gibco), 2 mM glutamine, 0.1 mg ml^{-1} penicillin-streptomycin; 1,000 U/ml LIF were added prior to use.

Antibodies

Antibodies raised against microtubule associated proteins (MAP2 a+b (ref. M1406) and MAP5 (ref. M4528)), neuro-filament 200 kDa (NF200, ref. N4142), synaptophysin (ref. S5768) and glial fibrillary acidic protein (GFAP, ref. G9269) antigens were purchased from Sigma. Antibody raised against the O4 antigen (ref. 1518925) was purchased from Boehringer Mannheim. Antibodies raised against neuronal cellular adhesion molecules (N-CAM; ref. 5B8), glutamic acid decarboxylase (GAD; ref. GAD6), and nestin (ref. Rat401) were purchased from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore and the Department of Biological Sciences, University of Iowa, Iowa City). All antibodies used in these experiments were mouse monoclonal antibodies, except for the antibody against GFAP (rabbit polyclonal).

In situ immunofluorescence

Cultures were washed once in Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) then fixed in 4% paraformaldehyde for 10 minutes. Prefixed cells were then dehydrated in 95% ethanol/5% acetic acid for 20 minutes at −20°C and washed three times for 15 minutes each in TBS. Cells were incubated overnight at 4°C with the first antibody (diluted at 10 μg/ml in TBS + 1% BSA), washed three times for 10 minutes each in TBS + 1% BSA, then incubated for 45 minutes with FITC-conjugated second antibody (dilution 1:100 in TBS + 1% BSA). Cells were mounted in Fluoprep (Biomerieux) containing an antifading reagent. The same protocol was used with the antibody raised against N-CAM except for the dehydration step which was ommitted.

Cresyl Violet staining

Cells were fixed and dehydrated as described for immunostaining, then washed twice in PBS for 10 minutes and incubated for 2 minutes in staining solution (0.25% Cresyl Violet, 0.8% glacial acetic acid, 0.6 mM sodium acetate). Cells were washed in PBS, 4 times 10 minutes each, then mounted for examination.

Detection of acetylcholinesterase activity

Detection of acetylcholinesterase (AchE) activity was performed according to a protocol using acetyl-choline iodide as substrate (Hardy et al., 1976). Incubation times ranging from 2.5-3 hours at 37°C were chosen to give optimal staining.

Electrophysiology

For experimentation, the culture dish was placed on the stage of an inverted microscope and the culture medium rapidly exchanged for the control saline solution (135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2.5. mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.2). The volume of the recording chamber was 1 ml and the bath solution was perfused by gravity at a rate of 15 ml/minute. Membrane voltage and whole cell currents were measured with borosilicate pipettes of 2-3 MΩ initial resistance by the perforated patch recording method using ionophore amphotericin B (Rae et al., 1991). The partition of the cell membrane by amphotericin B was achieved 10-15 minutes after the seal was established and series resistances, ranging from 8 to 12 MΩ, were electronically compensated before experimentations. Current and voltage clamp recordings were carried out using a patch clamp amplifier RK 400 (Bio-Logic). Pulse protocols were generated using the P-Clamp software (Axon Instruments, Burlingame). Membrane currents were digitized on line with an analogue-to-digital converter (Labmaster TM 40, Scientific Solutions Inc.) at 2 kHz (0.5 second steps) and 20 kHz (20 milliseconds steps), filtered at 1/3 of the sampling rate and stored on the hard disc of a computer through the P-clamp software. The leak current was not compensated. Tetrodotoxin (TTX) was added to the control solution to obtain a final concentration of 0.1 μM. 4-aminopyridine (4-AP; 5 mM, pH 7.2), was prepared just before use and tetraethylammonium ions (TEA; 20 mM) were substituted for Na^+ in the control solution. The pipette filling solution contained: 110 mM K aspartate, 20 mM KCl, 7 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, pH 7.2. Amphotericin B was prepared by sonication in dimethylsulfoxide (DMSO) and added into the pipette filling solution at a final concentration of 240 μg/ml. TTX, 4-AP, TEA, DMSO and amphotericin B were purchased from Sigma. All experiments were performed at room temperature (20-22°C).

RESULTS

Experimental procedure

At day 0, ES cells were plated at low density in bacterial grade Petri dishes (5×10^5 cells per 10 cm dish) in standard ES culture medium (without LIF) containing 1 μM all-*trans* RA (Fig. 1). At day 2, cell aggregates were collected and replated in tissue culture dishes (approximately 50 cell aggregates per 10 cm tissue culture dish) in ES cell culture medium (without both LIF and RA). Approximately half of the medium was changed once every week. Cell aggregates attached and proliferating cells spread and expanded progressively to form a monolayer of differentiated cells (Fig. 2A). After 4-5 days, cells showing the characteristic morphology of neuronal cells (soma and neurites) differentiated on top of this monolayer (Fig. 2B). These cells with a neuron-like morpholgy will hence be referred to as neuronal-like cells. They were observed either as isolated cells (Fig. 2B) or as aggregates of various sizes (Fig. 2A). They were specifically stained by Cresyl Violet and were also strongly positive for the N-CAM antigen (data not shown). At day 6, we estimated by visual examination that at least 10% of the cells exhibited this neuronal phenotype. From day 20 onwards, other differentiated cell types overgrew and neurone-like cells progressively disappeared.

Retinoic acid concentrations ranging from 10^{-9} to 10^{-5} M,

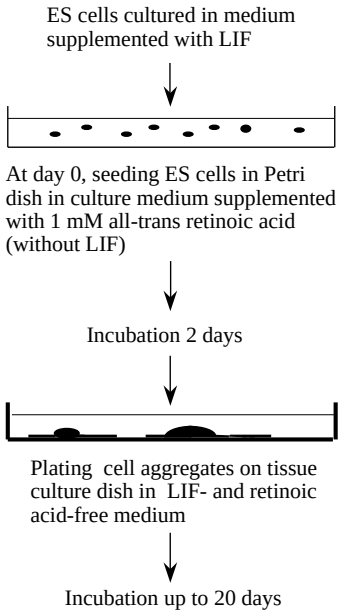

Fig. 1. Schematic presentation of the experimental protocol used for ES differentiation into neuronal cells.

and treatment with RA from 1 to 10 days, were tested for optimizing the extent of neuronal differentiation in the cultures. Two batches of serum (screened for ES cells culture) were used in differentiating media and they showed the same efficiency of neuronal differentiation. The frequency of neuronal-like cells was shown to occur with maximal efficiency when induction was performed with 1 μM all-*trans* RA for 2 days, as described above.

Antigenic characterization of neuron-glia precursors

At day 3-4, some small refringent cells could easily be observed (Fig. 3) on top of the cell monolayer. Within 2 or 3 days, cytoplasmic outgrowths developed from these cells and they progressively acquired a characteristic neuronal morphology. These cells were analysed by in situ immunostaining with an antibody raised against nestin, a specific antigen for precursor cells in the neural tube of 10-day-old rat embryos (Hockfield and McKay, 1985). Many of these small refringent cells were strongly stained with this antibody. These cells probably correspond to neuron-glia precursor cells. Other cells displaying a different morphology (flat non refringent cells) were also found to be positive for nestin antigen. These cells could be muscular precursor cells which are known to express

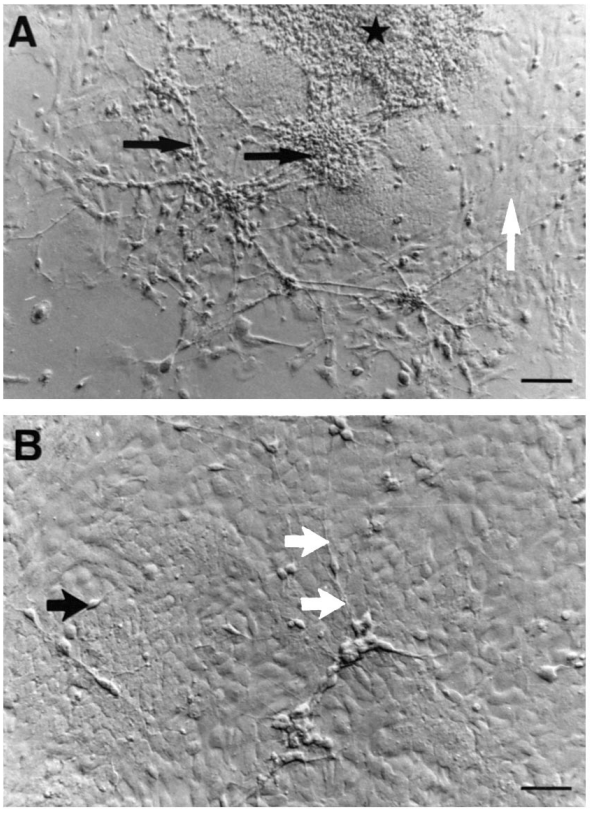

Fig. 2. Neuron-like cells in day 6 culture. (A) The remaining embryoid body (black star) plated at day 2, attached to the tissue culture dish. Proliferative cells spread around it in a cell monolayer structure (white arrow). Neuronal-like cells connected in a cellular network can be seen on top of the cell monolayer (black arrows). Bar, 80 μm. (B) These neuronal-like cells show the characteristic morphology of neuronal cells: soma (black arrow) and neurites (white arrows). Bar, 40 μm.

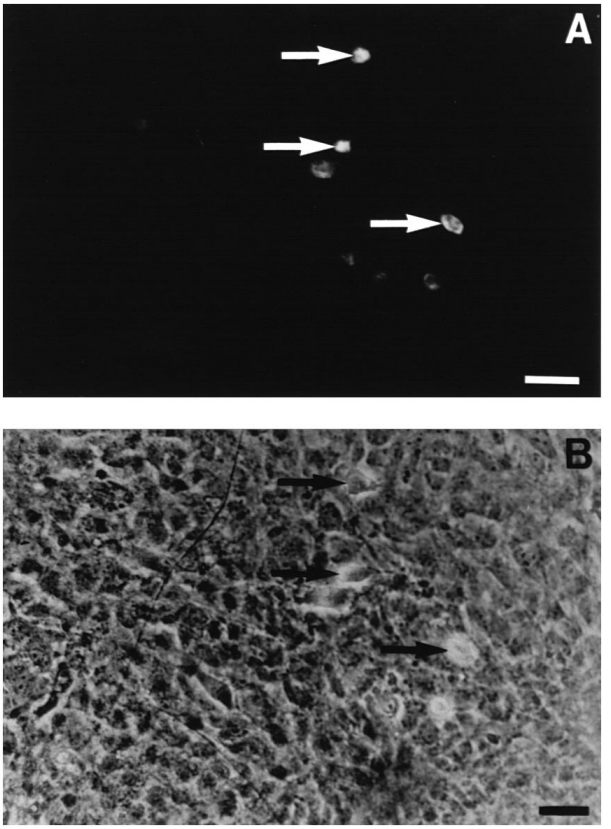

Fig. 3. Characterization of putative neuronal precursor cells (nestin-positive cells) in day 3 ES cell culture (1 day after replating on tissue-culture dish) by indirect immunofluorescence (FITC labelling). (A) Immunostaining with monoclonal antibody against nestin. The white arrows indicate positive cells. Bar, 30 μm. (B) Bright-field picture of the same field. Nestin-positive cells shown in (A) can be recognized on top of the cell monolayer as small and refringent cells (black arrows). Bar, 30 μm.

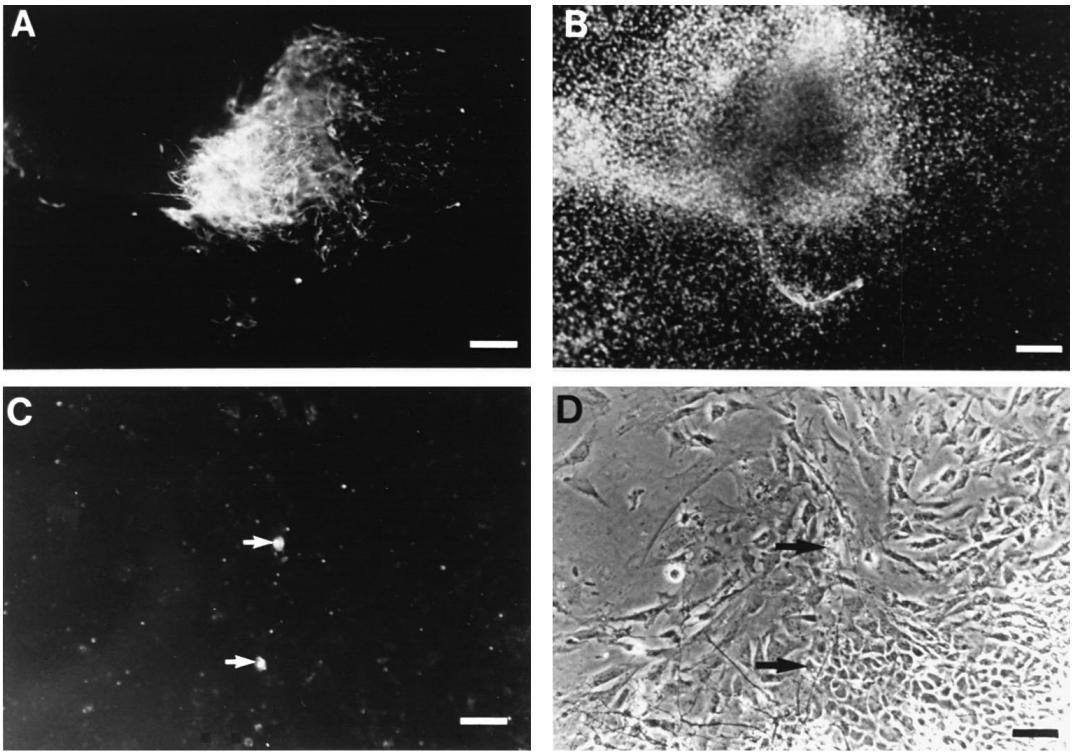

Fig. 4. Characterization of glial cells by indirect immunofluorescence (FITC labelling). At day 9, the culture was stained with an antibody raised against an astrocyte specific antigen GFAP (A,B) and against an oligodendrocyte specific antigen O4 (C,D). (A) Astrocytes (GFAP positive cells) are detected as an aggregate. Bar, 100 μm. (B) Hoechst staining of the field shown in (A). (C) Two oligodendrocytes (white arrows), O4 positive cells, can be detected among neuronal-like cells. Bar, 40 μm. (D) A bright field of (C).

a significant level of nestin (data not shown; Hockfield and McKay, 1985).

Antigenic characterization of glial cells

Oligodendrocytes are specifically stained by anti-O4 antibody (Schachner et al., 1981) whereas astrocytes are specifically stained by anti-GFAP antibody (Bignami et al., 1972). At day 9, GFAP-positive cells were clearly identified among differentiated cells (Fig. 4A,B). These astrocytes were usually detected inside cell aggregates, seldom as isolated cells. Some O4-positive cells (oligodendrocytes) were also detected, although with a much lower frequency (Fig. 4C,D). Oligodendrocytes were always found as isolated cells. In conclusion, ES cells can differentiate into both types of glial cells, astrocytes and oligodendrocytes.

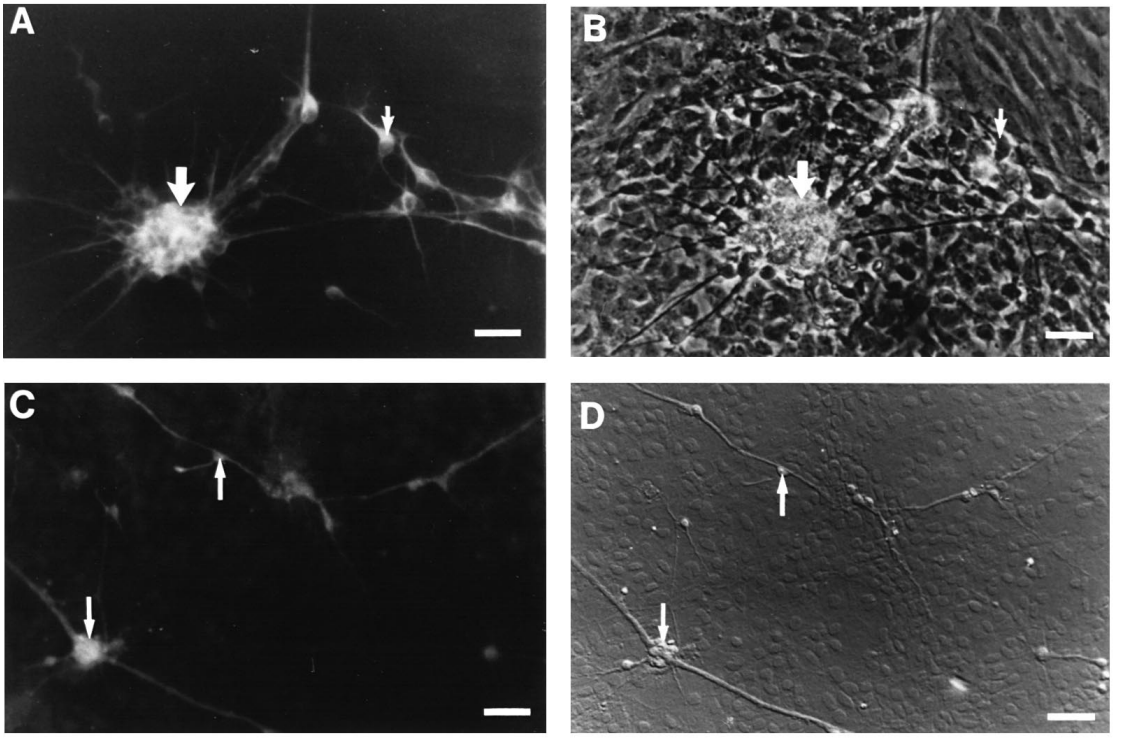

Fig. 5. Characterization of neurons by indirect immunofluorescence (FITC labelling) with antibodies raised against the neuron cytoskeleton protein MAP2 (a+b) (A,B) and against GAD (C,D). (B and D) Bright-field pictures of the fields shown, respectively, in A and C. (A) At day 8, the anti-MAP2 (a+b) antibody stained individual cells (small white arrow) or small cell aggregates (large white arrow). Positive cells displayed neuronal-like cell morphology. Bar, 30 μm. (C) At day 11, GAD positive cells can be detected (white arrows). Note that all the neuronal-like cells are GAD positive. Bar, 40 μm.

Antigenic characterization of neurons

To characterize post-mitotic neurons, expression of neuron-specific antigens (cytoskeleton proteins MAP2 (a+b) (Binder et al., 1986), MAP5 (Tucker and Matus, 1987), NF200 (Wood and Anderton, 1981) as well as synaptophysin a protein involved in synaptic function (Wiedenmenn and Franke, 1985) were tested using specific antibodies. At day 8, many cells were strongly positive with anti-MAP2 (Fig. 5A,B). The same staining pattern was observed using antibodies against MAP5 and NF200 antigens (data not shown). At day 10, some neuronal-like cells also expressed synaptophysin (Fig. 6A,B). Neurons positive for MAP2, MAP5, NF200 or synaptophysin were detected either as individual cells or in small aggregates. It is also noteworthy that the staining pattern of cytoskeleton proteins in these neurons was identical to the staining pattern observed in post-mitotic neurons in primary culture of embryonic cortex dissected out from E16 mouse foetuses (data not shown).

Characterization of acetylcholinesterase (AchE) and glutamic acid decarboxylase (GAD) positive neurons

The expression of enzymes involved in neurotransmitter metabolism such as acetylcholinesterase (AchE) (Small, 1990) and glutamic acid decarboxylase (GAD) (Chang and Gottlieb, 1988) was tested. In the CNS, several neuron types including cholinergic neurons contain AchE activity (Layer, 1983). In contrast, GAD is restricted to GABAergic neurons. AchE activity was detected, as early as day 8, in a subset of neuron-like cells (Fig. 6C). The intensity of the staining increased up to day 10. Similar results were obtained with the antibody raised against GAD, indicating the presence of GABAergic neurons among differentiated cells (Fig. 5C,D). Interestingly, AchE positive neurons always appeared as isolated cells, mixed with other neuron-like AchE-negative cells. In contrast, GAD-positive cells were found as clusters of positive cells, devoid of neuron-like GAD-negative cells. It is worth noting that these two neuron populations were identified at approximately the same frequencies, ranging from 10 to 20% of the total neuronal-like cell population.

Electrophysiological characterization of neurons

We further characterize electrophysiological properties of these neuronal-like cells. All the recordings were performed on isolated neuronal-like cells. The cell resting membrane potential measured in control solution by the perforated patch method ranged from –30 to –64 mV with a mean value of –43.8±11.2 mV (n=11 s.d.). Input resistance and cell capacity were 1.3±0.4 GΩ (n=6 s.d.) and 23.5±0.8 pF (n=3 s.d.), respectively. The injection of brief depolarizing current pulses (12 milliseconds duration) of increasing intensity produced membrane potential changes which triggered action potentials when the membrane depolarization reached –30, –25 mV from a membrane potential of about –60 mV (Fig. 7A). Depending on the cells, these action potentials peaked from –60 to +45 mV and their repolarizing phase was prolonged by a weak undershoot. They were insensitive to Cd^{2+} (300 μM), a blocker of voltage-dependent Ca^{2+} channels but were inhibited in the presence of 0.1 μM TTX, a blocker of voltage-dependent Na^{+} channels. The TTX-sensitivity of these action potentials indicates that their depolarizing phase is Na-dependent. In some cells (4 out of 11), only graded membrane depolarizations were obtained in response to current injections. Voltage clamp experiments showed that the total membrane current was dominated by a large transient outward current which overlapped a transient inward current. From a holding membrane potential of –90 mV, outward currents evoked for a voltage step to +50 mV consisted of a rapidly activating transient current followed by a maintained current (Fig. 7B). Activation potential of the transient outward current determinated from the current-voltage relation of the total membrane current was –60 mV. This current, which showed a strong outward rectification, was partially blocked by 5 mM 4-AP (Fig. 7B) and completely inhibited when the holding potential was made more positive than –60 mV. However, the peak and the decaying phase of this transient outward current were very variable from cell to cell. In some preparations (2 out of 8), the transient outward current was not detected. The transient outward current, sensitive to 4-AP and to holding potential is similar to I_A potassium current (Rudy, 1988) known to regulate interspike intervals during the rhythmic membrane firing of neurons (Connor and Stevens, 1971). The maintained outward current activated slowly at about –30 mV, rectified in outward direction and it was sensitive to 20 mM TEA (Fig. 7C). This maintained outward current likely corresponds to classic delayed potassium current. When 4-AP was added in the bath solution, a large transient inward current was detected (Fig. 7D). This transient inward current was reversibly inhibited by 0.1 μM TTX. Blocking effects of TTX reveals that the inward current is carried by Na^{+} and no experimental evidence suggests an

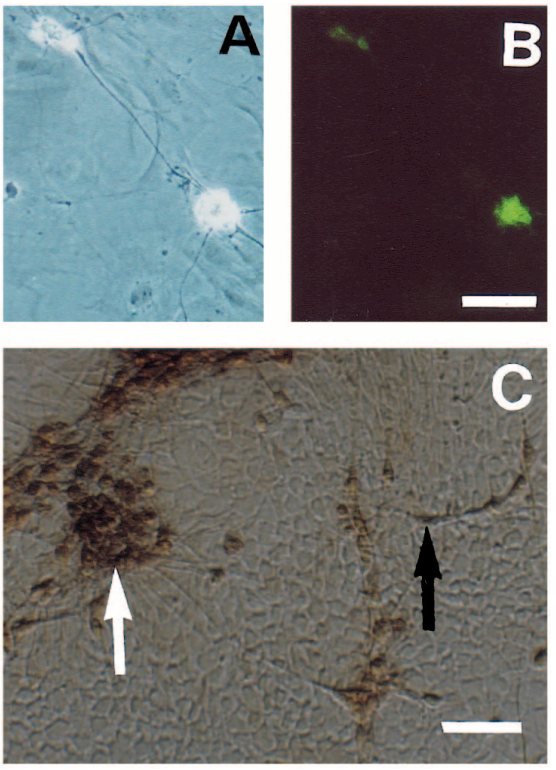

Fig. 6. Characterization of neurons by immunofluorescence against the synaptophysin protein (A,B) and by detection of AchE activity (C). (A) The bright-field picture of the field shown in (B). (B) At day 10, synaptophysin protein can be detected in neuronal-like cells. Bar, 60 μm. (C) At day 11, AchE positive cells are stained in brown (white arrow). Among the neuronal-like cells, AchE negative cells can be seen (black arrow). Bar, 40 μm.

eventual participation of Ca^{2+} in this inward current. Subtracting procedures between TTX-sensitive and insensitive currents indicated that the pure inward current activated at about −40 mV and was maximum at about 0 mV. Among these different ionic currents, the rapidly activating transient outward current can be involved in the modulation of the cell excitability. This current, which is more or less developed depending on the cells can overlap, to various degrees, with the Na^+ dependent transient inward current. Such a possibility would explain the variability of amplitude of action potentials and the absence of regenerative membrane responses detected in some cells.

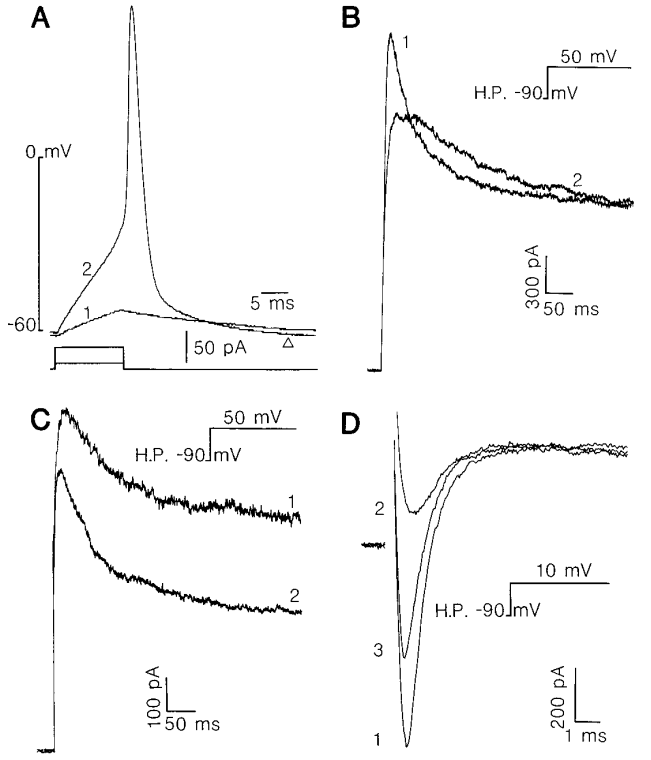

Fig. 7. Electrophysiological characterization of neurons obtained after 10 to 15 days of culture. (A) Membrane responses (upper traces) to depolarizing current pulses (lower trace) injected into a neuronal-like cell. A low current pulse evoked only a small deflection of the membrane potential (trace 1) while a higher current pulse triggered a fast overshooting action potential (trace 2). Measured at half amplitude, the duration of this action potential was 3 milliseconds and dV/dt was 16 V second^{-1}. Open triangle shows the undershoot. (B) 4-AP-sensitive outward current. Inset, stimulating protocol. In control solution (trace 1), a fast activating transient outward current was followed by a maintained outward current. 4-AP (5 mM) partially inhibited the peak of the transient outward current while the maintained outward current was insensitive to this substance (trace 2). (C) TEA-sensitive outward current. Inset, stimulating protocol. The total outward current (trace 1) was reduced by 20 mM TEA added to the external solution (trace 2). (D) TTX-sensitive transient inward current. Inset, stimulating protocol. The inward current (trace 1) was abolished by 100 nM TTX added to the control solution (trace 2). TTX-insensitive ionic current (trace 2) corresponds to the delayed outward current (trace 3: recovery). In this experiment, the transient outward current was blocked by 5 mM-AP continuously superfused in the bath solution. Note the time scale of this record compared with those illustrated in (B) and (C).

Time course of neuronal differentiation

The frequency of each neuronal cell type (nestin-, MAP2-, GFAP- and O4-positive cells, corresponding to neuron-glia precursor cells, neurons, astrocytes and oligodendrocytes, respectively) was estimated during the time course of differentiation (Fig. 8). Neuron-glia precursor cells (nestin-positive cells) first appeared at day 3 after the onset of differentiation. Their total number declined rapidly and could no longer be detected after day 10. Oligodendrocytes (O4-positive cells) and astrocytes (GFAP-positive cells) first appeared at day 5. O4-positive cells remained very low in number (approximately 1% of the total neuronal population), whereas GFAP-positive cells progressively increased in number between day 6 and day 9 (approximately 75% of total neuronal population at day 9). After day 9, the number of GFAP- and O4-positive cells progressively decreased and both types of cells were no longer detectable at day 20. Post-mitotic neurons (MAP2-positive cells) were first detected at day 5. At day 9, approximately 25% of the neuronal-like cells were positive for MAP2 antigens. In contrast with astrocytes and oligodendrocytes, the number of MAP2-positive cells still increased progressively up until day 15, then decreased slowly from day 15 onwards. At day 20, more than 90% of the neuronal-like cell population was neurons (MAP2 positive cells), whereas glial cells were no longer detectable. We hypothetized that culture conditions were no longer able to support the growth of proliferative astrocytes and oligodendrocytes, whereas they still promoted survival of post-mitotic neurons.

DISCUSSION

The data presented in this paper provide evidence that ES cells can differentiate into neuron-glia precursor cells and then into glial cells and functional neurons in vitro.

Voltage clamp data indicate that one sodium and two

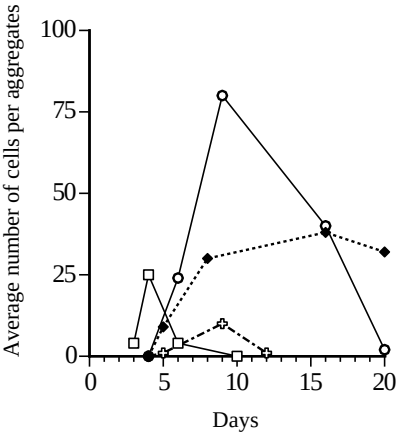

Fig. 8. Time course of the neuronal cell population development in culture. The curves indicate the relative cell number of each cell type during in vitro differentiation of ES cells. These cell numbers were scored per attached cell aggregate and values presented are means of 5-8 aggregates. (□) Precursor cells (nestin positive cells); (◆) neurons (MAP2 (a+b) positive cells); (○) astrocytes (GFAP positive cells); and (2) 10 times the real number of oligodendrocytes (O4 positive cells).

potassium ionic currents could be isolated on the basis of their voltage-dependence and pharmacological properties. Furthermore overshooting action potentials are triggered in response to current pulse injections. These electrophysiological properties are exactly what should be expected from neurons.

The synthesis of the GAD enzyme unambiguously revealed the presence of GABAergic neurons. In the CNS, cholinergic neurons contain AchE activity but many non cholinergic neurons also contain AchE activity (Layer, 1983). So we cannot conclude unambiguously the presence of cholinergic neurons in the culture. Interestingly, AchE positive neurons were usually identified next to negative neurons, whereas GABAergic neurons always appeared as more homogenous cell populations. It could be hypothesized that differentiation into mature GABAergic neurons specifically required a cell population effect. These two types of neurons were identified at approximately the same frequencies, ranging from 10 to 20% of the total neuronal-like cell population. This observation suggests that our differentiation protocol did not drive neuronal differentiation into one particular neuron cell type.

Neuronal differentiation of ES cells was shown to occur spontaneously after treatment with retinoic acid, in the absence of any added cytokines or specific growth factors. Differentiation was also shown to take place within a few days after replating. This is in contrast with what has previously been described with cardio-muscular and hematopoietic cell differentiations that were shown to take place in embryoid bodies after 8 to 10 days in culture (Wiles and Keller, 1991; Maltsev et al., 1993). Furthermore, hematopoietic differentiation was shown to be greatly enhanced in the presence of specific cytokines like Epo or IL-11 (Keller et al., 1993). It is also noteworthy that neuronal differentiation was easily obtained using two batches of foetal calf serum tested for their ability to promote the growth of ES cells and to prevent their differentiation. All these data suggest that neuron cell differentiation does not need complex conditions to occur in vitro at high efficiency. Interestingly, neural induction has recently been described as the derepression of a default state (Green, 1994). However, it is likely that conditioning of the medium by differentiating cells may play some role in enhancing neuron and glial cell differentiation. In vitro differentiation of astrocytes, oligodendrocytes, and neurons was shown to be strongly stimulated by cytokines or hormones when added to the culture medium (Almazan et al., 1986; Aizenman and de Vellis, 1987; Levi-Montalcini, 1987; Barres et al., 1994; Götz et al., 1994; Mizuno et al., 1994; Pinkas-Kramarski et al., 1994). Therefore, in our experiments, the conditioned medium probably supplied the minimal cocktail of cytokines requested to achieve proper differentiation of neurons and astrocytes. Only oligodendrocytes failed to differentiate at a detectable level from ES cells in our culture conditions. We believe that this failure does not stem from the inability of CGR8 cells to differentiate into oligodendrocytes because healthy transgenic mice were generated from these ES cells (unpublished data). Furthermore, the EC cell line PCC-7 was shown to differentiate into astrocytes and neurons but not into oligodendrocytes (Lang et al., 1989). Therefore, we hypothesize that in vitro culture carried out in the present study did not fulfill the requirements for proper oligodendrocyte differentiation. It would be of great developmental interest to test different cytokines or hormones in order to trigger differentiation into oligodendrocytes in our experimental system.

Neuron and glial precursor cells have been identified in the mouse, both following in vivo retroviral labelling (Price et al., 1991, 1992) and in primary cultures of embryonic cortex (Raff and Miller, 1983; Davis and Temple, 1994). Hockfield and McKay (1985) characterized neuronal precursor in the neural tube as nestin-positive cells. During muscle differentiation nestin positive cells were also described. However, the small and refringent cells (positive for nestin) that were identified at day 3-4 on top of the cell monolayer (Fig. 3) did correspond to neuron-glia precursor cells because they progressively acquired the characteristic morphology of neuron-like cells at later stages of differentiation. The time course of differentiation, appearance of nestin-positive cells at day 3-4 prior to neurons or glial cells, indicates that neuronal differentiation of ES cells in vitro mimicks in vivo differentiation, although the first steps of neuronal differentiation in vivo are not yet well characterized (Raff and Miller, 1983; Price et al., 1993; Davis and Temple, 1994). Unambiguous identification of precursors for neurons and glia among differentiating ES cells is of paramount importance because it will allow us to study the effects of cytokines on early differentiation of these precursors.

In conclusion, the data presented in this paper have established that ES cells can consistently, and with a high frequency, generate neuron-glia precursor cells, glial cells and fully functional neurons in vitro. It provides a unique model to analyze the effects of a wide spectrum of cytokines, growth factors or hormones on the earliest steps of neuronal development. Combined with this in vitro model, molecular techniques available for ES cells such as homologous recombination (Thomas and Capecchi, 1986; Mansour and Capecchi, 1988), inducible transgene expression (Whyatt et al., 1993) and gene trapping (Friedrich and Soriano, 1991) may contribute to the analysis of the molecular control of neuronal commitment and differentiation.

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