Neurons derived from PC12 cells have the potential to develop synapses with primary neurons from rat cortex

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Abstract. Neuron transplantation is considered to be a promising therapeutic method to replace functions lost due to central nervous system (CNS) damage. However, little is known about the extent to which implanted neuron-like cells can develop into mature neurons and acquire essential properties, and especially formation of synapses with host neurons. In this investigation we seeded PC12 cells labeled with GFP into primary cultured neurons isolated from rat cerebral cortex to build a co-culture system, and then induced the PC12 cells to differentiate into neuron-like cells with NGF. Seven days later, we observed the relationship between the PC12-derived neurons and primary neurons using FM1-43 imaging and immunoelectron microscopy, and found that GFP-labeled neurons could form typical synapses with host primary neurons. These observations showed that immigrant neurons differentiated from PC12 cells could develop into mature neurons and could form intercellular contacts with host neurons. Both the immigrant and host neurons could construct neuronal networks in vitro.

Key words: neural transplantation, co-culture, PC12 cells, synapse, laser scanning confocal microscopy, electron microscopy
INTRODUCTION

Cell transplantation is an experimental approach to repairing brain injury after a stroke and also represents a possible strategy to restore brain functions in neurodegenerative disorders such as Parkinson's and Huntington's diseases (Gross 2000, Sanberg and Brundin 1999, Saporta et al. 1999). Various cell types are under investigation in experimental studies, of which the most promising cell sources for neuronal transplantation are immature neurons or their progenitors, including fetal tissue, cell lines and stem cells (Date et al. 2000, Flax et al. 1998; Lindvall et al. 1990, Lundberg et al. 1997, Ono et al. 1997, Park et al. 1999). Neuronal plasticity is now widely accepted as a fundamental mechanism, involving not only the rearrangement of neurons and their interconnections, but also the formation of new neural cells in animals and humans during their entire lifespan (Finley et al. 1996, Gross 2000). It is possible that the transplanted neurons integrate into neuronal networks composed of host neurons and repair the lost functions caused by neurodegeneration or CNS injury. While much interest has been focused on the long-term survival and differentiation of transplanted neurons, less attention has been paid to the extent to which implanted neurons can give rise to mature neurons and develop essential properties, especially an ability to form synapses connected with host neurons.

The PC12 cell line, a rat pheochromocytoma cell line, has been extensively used as a tool for studying the function of neurotrophic factors and neuronal differentiation because these cells can be induced to differentiate into neuron-like cells by NGF (Greene and Tischler 1976) or other factors (Arsenijevic and Weiss 1998, Sucher et al. 1993, Sugita et al. 1999, Tyson et al. 2003). The transplantation of PC12 cells into the striatum represents one potential means for the treatment of Parkinson's disease (Hefti et al. 1985). Studies have revealed that PC12 cells survive and continue to release dopamine up to 12 months after transplantation (Ono et al. 1997), but it is not clear whether the implanted PC12 cells can develop contacts with host neurons. In this study, we established an in vitro co-culture system to investigate if the neurons derived from PC12 cells could develop synaptic connections with host primary cultures.

METHODS

PC12 cell culture and transfection

Rat pheochromocytoma PC12 cells were maintained in plastic flasks in a DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% horse serum and 5% fetal bovine serum (Gibco, NY) in a humidified atmosphere with 5% CO₂, at 37°C. The pEGFP-N1 vector (Clontech) was transfected into the PC12 cells using lipofectAMINE 2000 reagent (Invitrogen), and stable transfectants bearing the EGFP expression unit were isolated under the pressure of G418 (Sigma, 500 µg/ml). EGFP positive cells were visually confirmed under a confocal microscope in separate cultures (Yoon et al. 2002).

Isolation of neurons from the cortices of newborn rats

Brains were taken from newborn Wistar rats. Under an anatomical lens, the cerebral cortex was separated and sheared into scraps after removal of membranes. The scraps were digested with 0.25% trypase at 37°C for 60 min, then washed twice with culturing medium. After agitating the tissue was dispersed into single cells and subsequently cultured in DMEM supplemented with 10% horse serum and N3 in a 35 mm dish in an incubator containing 7.5% CO₂ and 92.5% air at 37°C. Four days after the initial seeding, 0.1 µM Ara-c was added to the medium and the cells were cultured for another day to kill the dividing cells. Then Ara-c was removed by changing the medium completely. Verification of the culture composition under a phase contrast microscope revealed predominant presence of neurons, each with a clear nucleus and nucleolus, and long processes, forming a dense network.

Co-culture and differentiation of PC12 cells

Prepared PC12 cells were rinsed twice and harvested. Then the PC12 cells transfected with the pEGFP-N1 vector were seeded into primary neurons derived from the rat cortices. After seeding, 25 ng/ml NGF was added into a medium containing DMEM supplemented with 1% horse serum. To be induced to differentiate into neurons, the cells were incubated at 37°C in an atmosphere of 7.5% CO₂ and 92.5% air. Four days later 0.2 µM Ara-c was added to the medium to kill mitotic neurons. The co-culture was maintained for at least another 7 days.
**FM1-43 fluorescent imaging**

FM1-43 (N-(3-triethylammoniumpropyl)-4-(4- (dibutylamino)styryl) pyridinium dibromide, Molecular Probes) is a dye used to study cell membrane trafficking. Three properties make FM dyes useful for studying exocytosis, endocytosis, and endosomal trafficking: (i) the dyes partition reversibly in membranes; (ii) the dyes do not 'flip flop' and so are never free in the cytoplasm; (iii) the dyes are far more fluorescent in membranes than in water. The excitation wavelength of FM1-43 is 510 nm, and that of emitted light is 455 nm. Measurements were performed at 37°C in modified Krebs-Ringer bicarbonate solution comprising 130 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM HEPES, 2.0 mM NaHCO₃, 1.5 mM CaCl₂, and 5.5 mM glucose, equilibrated with O₂/CO₂ (95:5, v/v) (KRBS) (Pouli et al. 1998). Synaptic terminals were loaded with FM1-43 (10 μM) using a depolarizing stimulus (50 mM external K⁺, 60 s) to induce exocytosis. All the cells were fixed by 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes.

Confocal imaging was performed on a confocal laser scanning system (Radiance 2100TM, Bio-Rad, USA) attached to a TE300 microscope (Nikon, Japan) using a 100× Nikon Plan Fluro oil immersion objective. The GFP was excited using a 488-nm argon/krypton laser and detected with a 515–530-nm band pass filter. The FM-143 was excited using a 543-nm argon/krypton laser and detected with a 590–570 nm band pass filter. Images were acquired using a single scan and analyzed by LaserSharp2000 software (Bio-Rad, USA). The aperture size was optimized to increase signal-to-noise ratio. Images were digitally enhanced to identify active synaptic terminals, which appeared as fluorescent “puncta” (Trudeau et al. 1996).

**Immuno-electron microscopy**

Under a phase-contrast microscope the co-cultured neurons were marked and fixed for electron microscopic study. The culture cells were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde in a 0.1 M phosphate buffer for 30 min, followed by post-fixation with 0.5% osmium tetroxide in a 0.24 M phosphate buffer for 60 min. The fixed cells were dehydrated through a series of graded ethanol (50%, 70%, 90%, 100%), each for 5 min. Finally, the cells were infiltrated in 100% ethanol with Epon 812 (without DMP) for 20 min. They were infiltrated in Epon 812 (containing DMP) for 12 hours at room temperature and then embedded in resin. After the embedding medium was changed, the embedded cells were put in a thermostat to accelerate solidification. This process took 12 hours at 35°C, 12 hours at 45°C, and 24 hours at 60°C successively. Immunogold labeling was performed using 10 nm diameter colloidal gold particles, which were coupled to anti-rat IgG. The nickel grids were first treated with 8% sodium hypohidrate for 20 min at room temperature, followed by three washes in 0.05 M TBS, pH 7.2, each for 5 min. The sections were then immersed in antibody solution which contained 5% bovine serum albumin and GFP antiserum (Clontech, dilution: 1:200). The nickel grids were washed three times with TBS, followed by incubation with colloidal gold-coupled IgG (1:10) for 50 minutes at room temperature. They were then washed again another three times in distilled water. Finally, they were counterstained with saturated uranyl acetate and lead citrate, and then observed with an electron microscope (Philips 120).

**RESULTS**

**Choice of PC12 cells labeled with GFP**

To label the PC12 neuronal cells, undifferentiated PC12 cells were transfected with pEGFP-N1 vector so that the cells could stably express EGFP. Forty-eight hours after transfection, about 5% PC12 cells could be visually confirmed as EGFP positive under a fluorescence microscope. About 30% of the labeled cells expressed EGFP strongly, while the remaining cells expressed EGFP weakly. After two weeks’ culture under the pressure of G418, EGFP positive cells became dominant and grew into clones, remained polygonal with an undifferentiated morphology and without any neural processes.

**Isolation of primary cells**

The primary cells mainly comprised neurons and astrocytes. Most of the neurons had a clear nucleus and a nucleolus. They also had a single axon and one or more dendritic branches emerging from various locations of the cell body, and their processes became very long, forming a dense network. The other cells were flat astrocytes, which lay under the neurons and had short, thick processes.
Co-culture of primary neurons and PC12-derived neurons

EGFP positive PC12 cell clones were picked out before co-culture to ensure that each implanted PC12 cell could express EGFP (Fig. 1b, h, k). One day after 25 ng/ml NGF was added to the co-culture system, neurites began to grow out from the PC12 cell bodies. The neurites became longer and the number of neurites increased, suggesting that the PC12 cells had differentiated to neuron-like cells. In the differentiated neuron-like PC12 cells, cell bodies and main processes were EGFP positive, viewed under a fluorescence microscope. Observed with laser confocal scanning microscopy, EGFP labeled PC12 neuronal cells in the co-culture system dispersed in the network were composed of neurons and their processes. After 7 days of co-culture, the network was stained with Dil (Fig. 1a). Figure 1b shows an EGFP positive PC12 cell in the co-culture system. Fig 1c is the merged image of Fig. 1a and Fig. 1b, showing a PC12 cell in the network of primary neurons.

FM1-43 imaging

Synaptic terminals were loaded with FM1-43 (10 μM) using a depolarizing stimulus (50 mM external K+, 60 s) to induce exocytosis. In this investigation, the labeled synaptic terminals could be seen on the surface of primary perikarya as well as on the surface of neurons differentiated from PC12 cells.

In the co-culture system intercellular contact formation between neurons derived from PC12 cells (EGFP labeled) and primary cortex neurons occurred within one week, as indicated by staining with FM1-43. A raw FM1-43 image (Fig. 1g) shows vesicles of a synapse stained by FM1-43 in a cell body and its neurites, and a raw GFP image (Fig. 1h) shows that the cell was EGFP-. A merged image (Fig. 1i) suggested that the cell was a neuronal PC12 cell. The other three images (Fig. 1j,k,l) show vesicles of synapses stained by FM1-43 in another neuronal PC12 cell body and its neurites. As a comparison, Fig. 1d shows vesicles stained by FM1-43 in a cell body and its neurites, and Fig.1e shows that the cell was EGFP negative. The merged image (Fig. 1f) shows that the cell was a primary neuron.

Fig 1. Fluorescence images showing the relationship between primary neurons and migrated neurons derived from PC12 cells. Image (a) shows host primary neurons labeled with Dil as red. A substantial number of perikarya and their processes were noted. A green fluorescence labeled neuron with elaborated processes is the neuron differentiated from PC12. A substantial number of varicosities were noted on the processes. Image (c) presents a merged image showing the relationship between the primary neurons and the migrated neurons derived from a PC12 cell. Image (d) shows a primary neuron and its processes labeled by FM1-43. Many red fluorescence spots form the outline of the neuron and the processes. Image (e) shows the same neuron showing no labeling for GFP. Image f shows the merged image. Another FM1-43 positive neuron is shown in image (g). The same neuron derived from a PC12 cell was labeled by stronger green fluorescence (h). On the merged image (i), two fluorescence labels can be seen clearly. Images (j)–(l) show a double process labeled by two kinds of fluorescence. Many red fluorescence spots [arrow in (j)] and green labeling processes (k) were noted to be superimposed (l). Scale bars are 50 μm.
Synapse structures under electron microscopy

Under an electron microscope, neurons differentiated from PC12 cell could be distinguished from primary ones due to the chromaffin granules in their perikarya and processes (Fig. 2a–b). Comparing the terminal swelling of the two kinds of neurons, there were obvious differences (Fig. 2c–f): (i) microtubules in axons of PC12 neuronal cells were thicker and straighter than those in axons of primary neurons; (ii) axons and terminal varicosity swellings of the PC12 neuronal cells were darker than those of the primary neurons; (iii) the terminal swellings of PC12 neuronal cells were much more regular and bigger than those of primary neurons.

In this study we observed three types of typical synapses and synapse-like structures. They were between either two primary neurons, two neurons differentiated from PC12 cells, or one primary neuron and one PC12-derived neural cell. We paid special attention to the last type of synapse since it suggested the existence of a new, potentially functional relationship between these two neurons. Since PC12 derived neuron-like cells were sprinkled into the network composed by primary neurons and their processes (Fig. 1c), connections were formed predominantly with primary neuronal processes and also with the synapses themselves. We oriented primary neurons alone in the cluster of EGFP positive PC12 neuronal cells to search for synapses between primary neuronal soma and processes of PC12 neuronal cells. These synaptic structures were indistinguishable from those between primary neurons in terms of size of active zones and number of vesicles. In these synapse structures, presynaptic and postsynaptic membranes, synaptic clefts and vesicles could be found and all the synaptic vesicles were round and clear. Both asymmetric and symmetric synapses were observed.

Fig 2. The fine structures of host primary and immigrant neurons and their relationship. Image (a) shows the close relationship between a primary perikaryon and a processes derived from a PC12 cell, characterized by its high electron density structure. A synapse was established between these cells [see inset, shown in boxed section of image (a)]. Image (b) shows that a perikaryon of a PC12 cell, characterized by its chromaffin granules, was in close contact with a varicosity (asterisks) from a primary neuron. A synapse-like structure (arrow) was noted. Many dense chromic grains were seen in the perikaryon. A varicosity was observed in close contact with this soma with chromic grain (c). Image (d) shows high magnification of the box appearing in image (c), to illustrate this close relationship. Images (e) and (f) show the fine structure and synapse between a varicose-derived PC12 cell and primary perikaryon. Many rough endoplasmic reticula, free ribosomes, and mitochondria were noted in the cytoplasm of the perikaryon. A varicosity was observed in close contact with this perikaryon [see box in image (e)]. Image (g) shows a complex synaptic structure among the primary neuronal processes and a varicosity differentiated from PC12 cells. At least three synapses (arrows and a box) were seen to form. Typical parallel arrangement of neurotubules is seen. At high magnification [in the box in image (g)], a typical Gray I synapse (including presynaptic and postsynaptic membrane, synaptic cleft and vesicles) was exhibited. The presynapse varicosity was also labeled with colloidal gold particles ([see box and inset in image (h)]. However, we did not see any colloidal gold particles in postsynaptic structures. Scale bars are 500 nm.
Immunoreactive (IR) EGFP-IR

Because chromic grains were located mainly in cell bodies and near processes of neurons differentiated from PC12 cells, it was difficult to identify finer processes. Here, we employed immunogold-labeled antibodies against GFP to display these fine processes and their relationship with primary neurons. We detected that some typical synapses or synaptic structures occurred between PC12-derived neuronal cell processes (labeled with colloidal gold particles) and primary neuron terminal swellings (Fig. 2g–h). In these synaptic structures, presynaptic and postsynaptic membranes, synaptic clefts and vesicles could be found and all the synaptic vesicles were round and clear.

DISCUSSION

In this study, we have demonstrated by FM1-43 imaging and electron microscopy, that neuron-like cells derived from PC12 cells have the potential to develop synapses by connecting with primary neurons when provided with a permissive transplantation environment. The manipulation of the co-culture system allowed a precise analysis of neuronal maturation and connection formation of transplanted neurons, and their relationships with host neural cells.

Differentiating and constructing synapses

For a long time many research groups have been searching for neural cells for transplantation to restore brain functions lost due to injuries or neurodegenerative disorders (Date et al. 2000, Flax et al. 1998, Lindvall et al. 1990, Lundberg et al. 1997, Ono et al. 1997, Park et al. 1999, Ryder et al. 1990). The ideal neural cells for transplantation should have these features: (i) the ability to remain undifferentiated and to self-renew; (ii) the ability to change phenotype to neural cells; (iii) be genetically manipulatable for transplantation of modified cells into the brain. The last feature represents a promising strategy for the expression of specific neurotrophic factors and neurotransmitter-synthesizing enzyme (Henningson Jr et al. 2003, Singh 2001, Wyman et al. 1999, Zhang et al. 2003). Our investigation demonstrated that neuron-like cells derived from cell lines, such as the PC12 cell, have the potential to develop functional synapses by connecting with primary neurons when provided with a permissive transplantation environment. In the co-culture system composed by PC12 cells and primary neurons, the PC12 cells induced by NGF outgrew neurites and formed synapses with primary neurons. These data suggest that differentiated neurons derived from undifferentiated precursors could be used to generate high-throughput functional neurons, which can serve as a reproducible source of cells for reconstructive therapies where synapse function is necessary.

Comparison of synaptic structures

Synapses formed between PC12-derived neuronal cell bodies and processes of primary neurons, and those between PC12-derived neuronal cell processes and cell bodies of primary neurons, were indistinguishable from those between primary neurons in terms of size of active zones and number of vesicles. However, there were some ultrastructural differences between terminal swellings. The PC12 cell line is able to form cholinergic synapses with a clonal cell line of skeletal muscle origin (Schubert et al. 1977). Our study showed that the vesicles of synapses were round clear vesicles but not large dense-core vesicles.

Environment in co-culture system

In the co-culture system, there should be many kinds of factors that affect differentiation, maturation and/or synapse formation of implanted neurons, many of which are produced by host cells. It will be helpful to manipulate the implanted neurons’ function if we make the role of these factors clear (Kirschchenbaum and Goldman 1995, Meher et al. 1993, Noma et al. 1999, Shetty and Turner 1998, Vicario-Abejon et al. 1995). Interestingly, we noticed that when the PC12 cells alone were induced by NGF, they would differentiate into neurons with simple and immature morphology. When the co-culture system had a certain number of glial cells in it, the PC12 cell-derived neurons showed complex and mature appearance with many more and longer processes. This might contribute to the environment supplied by primary neural cells, especially by glia. Data have demonstrated that astrocytes increase the number of functional synapses of neurons by sevenfold and they are required for synaptic maintenance in vitro (Ullian et al. 2001). Glial cells are important to synaptic strength at excitatory synapses and may play
a role in synaptic plasticity and modulating responses to neural injury (Beattie et al. 2002, Fields and Stevens-Graham 2002).

CONCLUSION

In the present study, the co-culture system that retained glial cells reconstructed the environment of the brain, which suggests that when PC12 cells would be transmitte into the brain, they could successfully differentiate into neuron-like cells and have the potential to develop functional synapses with host neurons. These facts indicate the possible significance of PC12 cells in clinical application to treat neural diseases.

REFERENCES


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