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NOTE

Accumulation of neurons differentiated from mouse embryonic stem cells in particular areas of culture plate surface

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Nanoscale magnetic beads coated with nerve growth factor (NGF) allow us to accumulate neurons differentiated from mouse ES cells in a selected area of the culture plate surface using a magnet. Neurons with neurite outgrowths within a particular area expressed TrkA and incorporated beads in the soma.

[**Key words:** ES cell; Accumulation; Neuron; NGF-coated nanoscale magnetic bead]

Mouse embryonic stem (ES) cells have a pluripotent ability to differentiate into a variety of cell lineages including neurons *in vitro*. The *in vitro* differentiation of ES cells provides new perspectives for studying the cellular and molecular mechanisms of early development and the generation of donor cells for transplantation therapies (1). Therefore, it is valuable to control the differentiation of ES cells into neurons and important to collect neurons differentiated from ES cells to realize potential applications in neuroscience and regenerative medicine. We previously reported that a chick dorsal root ganglion (DRG) conditioned medium (CM) promoted the differentiation of ES cells into neurons (2). We also found that the percentage of neurons differentiated from ES cells was approximately 50%. The 40–60% of neurons that differentiated from the ES cells were mainly motor neurons as target cells of DRG neurons (sensory neurons) (3).

Because the differentiation of ES cells produces various types of cells, we needed to efficiently isolate neurons from differentiated cells. Hedlund et al. used fluorescence-activated cell sorter (FACS) for purification of dopamine neurons derived from embryonic stem cells to provide a functional cell population for transplantation while eliminating the risk of teratoma formation (4). Jüngling et al. reported an immunoisolation procedure to purify neurons from *in vitro* differentiated mouse ES cells using an antibody against the neuronal cell adhesion molecule L1 (5). Nanoscale magnetic beads have been used for isolating high-purity retinal endothelial cells (6), rat retinal ganglion cells (7), choriocapillary endothelial cells (8), and specific hybridoma clones (9). Tucker et al. described a procedure using magnetic beads from Dynal to select and plate viable populations of neurons based on the expression of specific cell surface markers (10). We have already reported the utilization of neurotrophin-coated magnetic beads immobilized by a magnet to culture DRG neurons in a particular area of the culture plate surface (11). In neuronal development, growth, regeneration, and repair process, neurotrophins such as nerve growth factor (NGF) are able to facilitate neuron survival and neurite outgrowths. Therefore, we investigated a way of isolating neurons from a variety of differentiated cells in a selected area of the culture plate surface by using nanoscale magnetic beads (250 or 50 nm in diameter) coated with NGF and an 11-mm magnet. Neurons accumulated in a selected area of the culture plate may be used for transplantation and drug screening as functional cells. In addition, protein-coated nanoscale magnetic beads may be applicable to microintegrated systems such as micrototal analysis systems (μTAS) and biosensors with parallel detection capability because the beads allow the cultivation of differentiated neurons in a selected area of the culture plate surface by using a magnet.

Glutaraldehyde-activated nanoscale magnetic beads with a nominal diameter of 250 nm (al250; Clemente Associates, Madison, CT, USA) or 50 nm (al50; Clemente Associates) were purchased ready for the covalent coupling of proteins. Ten microliters of glutaraldehyde-activated nanoscale magnetic bead solution and 15 μl of 10 μg/ml NGF solution (2256X; Techne, Minneapolis, MN, USA) or 15 μl of 10 μg/ml bovine serum albumin solution (BSA; 1024; Irvine Scientific, Santa Ana, CA, USA) were mixed for 30 s in a bath sonicator (US-2; Chuourikaki, Nagoya) and incubated for 2 h at room temperature. After the NGF-coated beads were allowed to settle by using a magnet, the supernatant was removed and the beads were washed four times with 75 μl of phosphate-buffered saline (PBS) by sonication. They were suspended in 1 ml of PBS by sonication. The binding of NGF to the beads was confirmed by ELISA (data not shown). BSA-coated magnetic beads were also prepared in the same manner as NGF-coated magnetic beads.

To differentiate mouse ES cells (129SV; Dainippon Pharmaceutical, Osaka) into neurons, one ES cell colony along with DMEM/F-12K

Abbreviations: BSA, bovine serum albumin; CM, conditioned medium; DRG, dorsal root ganglion; ES, embryonic stem; FITC, fluorescein isothiocyanate; NGF, nerve growth factor; PBS, phosphate-buffered saline.

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medium supplemented with 5% DRG-CM was plated per well on a gelatin-coated 96-well assay plate (3860-096; Iwaki, Chiba) and cultured for 12 d at 37 °C in 5% humidified CO_2, as described in detail elsewhere (3). ES cell colonies were washed twice with PBS and trypsinized with 20 μl of 0.25% trypsin/1 mM EDTA solution (SM-2003-C; Chemicon International, Temecula, CA, USA) for 2 min. After adding 100 μl of DMEM/F-12K medium to each well, differentiated ES cells were dispersed by pipetting and collected in a 15 ml tube. After being washed with DMEM/F-12K medium, the differentiated ES cells were suspended in 1 ml of DMEM/F-12K medium. To remove cell aggregates, the dissociated ES cell suspension was filtered through a 40-μm pore nylon mesh. The numbers of viable cells were counted using the trypan blue exclusion method and a hemocytometer. The percentages of viable cells were in the range of 56% to 82%.

After three 11-mm magnets were glued at intervals onto the reverse side of a gelatin-coated 60-mm culture dish (150288; Nalge Nunc International, Roskilde, Denmark), 10 μl of NGF-coated 250-nm magnetic bead solution and 10 μl of BSA-coated 250-nm magnetic bead solution was placed onto the two magnet areas, followed by the addition of 5 ml of DMEM/F-12K medium containing differentiated ES cells (approximately 4.5×10^5 cells). After 2 days of cultivation, the densities of neurons with neurite outgrowths in the areas of the 11-mm magnets (95 mm^2) and those in the peripheral areas of the 11-mm magnets (annulus, 106 mm^2) were measured by using an inverted microscope (IX50; Olympus, Tokyo). Fig. 1 shows the densities of neurons with neurite outgrowths in the areas and the peripheral areas of the 11-mm magnets. The density of neurons with neurite outgrowths in the area of the magnet was 0.54 cells/mm^2. The peripheral area was 0.13 cells/mm^2 of a background level. We could observe a lot of neurons with neurite outgrowths in the area of the magnet, because the area of the magnet was covered with NGF-coated magnetic beads. But a small number of neurons with neurite outgrowths and a lot of non-neuronal cells adhered in the peripheral area. On the other hand, the density of neurons with neurite outgrowths using BSA-coated nanoscale magnetic beads was at a background level in the area of the magnet. In the case of 50-nm beads, neurons with neurite outgrowths were also accumulated in the magnet area.

We also used one area of an 11-mm magnet glued onto the reverse side of a gelatin-coated 35-mm culture dish (153066; Nalge Nunc International) and another area of 11 mm in diameter without beads and magnet in the gelatin-coated 35-mm culture dish. Ten microliters of NGF-coated 250-nm magnetic bead solution was placed onto the magnet area, followed by the addition of 2 ml of DMEM/F-12K medium containing differentiated ES cells (approximately 1.0×10^5 cells). After 2 days of cultivation, the densities of neurons with neurite outgrowths in the area of the 11-mm magnet with magnet beads and the area of 11 mm in diameter without beads and magnet were measured by using an inverted microscope as shown in Fig. 2. The density of neurons with neurite outgrowths was 5.0 cells/mm^2 in the magnet area with NGF-coated magnet beads or 0.17 cells/mm^2 in the area of 11 mm in diameter without beads and magnet. On the other hand, the density of neurons with neurite outgrowths was 0.33 cells/mm^2 in the magnet area with BSA-coated magnet beads or 0.028 cells/mm^2 in the area of 11 mm in diameter without beads and magnet. The density of neurons with neurite outgrowths was 0.042 cells/mm^2 in the magnet area without magnet beads. We could efficiently accumulate a lot of neurons in the magnet area with NGF-coated magnet beads.

These results show that neurons could be isolated from various types of cells differentiated from ES cells and cultured in a particular area of the culture plate surface by using both NGF-coated nanoscale magnetic beads and a magnet.

Although cells are generally isolated by using FACS (4) or collection devices together with magnetic beads (6–10), cells can be accumulated and cultured in a selected area of the culture plate surface according to our method. We can easily obtain living neurons differentiated from ES cells. On the other hand, it is known that human ES cells are vulnerable to apoptosis upon cellular detachment and dissociation. They undergo massive cell death particularly after complete dissociation, and the cloning efficiency of dissociated ES cells is generally $\leq$1% (12). Therefore, when differentiated, mouse ES cells were dissociated by trypsin and plated in a culture dish, a lot of cells might undergo cell death by apoptosis. It is necessary to prevent apoptosis for accumulating a lot of neurons in the magnet area.

NGF induces cell differentiation and neurite outgrowth by binding with and activating the TrkA (NGF receptor). Therefore, we investigated the expression of TrkA protein in a differentiated cell by immunocytochemistry. The differentiated cells accumulated on a gelatin-coated microscope cover glass (12-545-82; Fisher Scientific, Pittsburgh, PA, USA) using 250-nm beads were washed three times for 5 min each in PBS and fixed with 4% paraformaldehyde for 30 min at room temperature and 90% methanol for 30 min at 4 °C. After being washed three times for 5 min each in PBS, the cells were incubated for 3 min at −80 °C. The cells were then washed again three times for 5 min each in PBS and then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used for

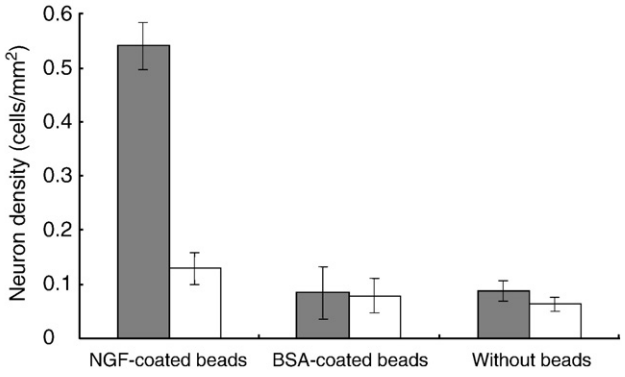

FIG. 1. Accumulation of neurons differentiated from ES cells by using NGF-coated nanoscale magnetic beads and a magnet in a gelatin-coated 60-mm culture dish. Differentiated cells were inoculated at approximately 4.5×10^5 cells. Closed bars, magnet area; open bars, peripheral area. Values are means of three replicates ± SE.

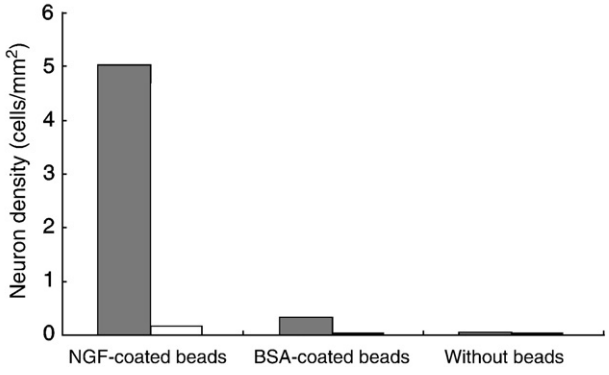

FIG. 2. Accumulation of neurons differentiated from ES cells by using NGF-coated nanoscale magnetic beads and a magnet in a gelatin-coated 35-mm culture dish. Differentiated cells were inoculated at approximately 1.0×10^5 cells. In the case of NGF-coated beads and BSA-coated beads; closed bars, the area of 11-mm magnet with magnet beads; open bars, the area of 11 mm in diameter without magnet beads and magnet. In the case of without beads; closed bar, the area of 11-mm magnet without magnet beads; open bar, the area of 11 mm in diameter without magnet beads and magnet.