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ORIGINAL ARTICLE

Induction of Neuron-Specific Enolase Promoter and Neuronal Markers in Differentiated Mouse Bone Marrow Stromal Cells

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Abstract

Mesenchymal stem cells in the adult bone marrow are differentiated to connective tissue, muscle, bone, cartilage, and fat cells. Recent studies in cultures, animal models, and humans demonstrated the plasticity of these cells and their capacity to express neuronal markers. However, questions were raised as to whether the neuronal phenotypes reflect transient changes or even fusion with neurons. In this study, we induced the differentiation of mouse stromal cells to neuron-like cells and observed the activation of the tissue-specific promoter of neuron-specific enolase (NSE). We used transgenic (Tg) mice that carry the antiapoptotic human *bcl-2* gene, expressed only in neurons under the NSE promoter. Some previous studies have indicated that the transgene induces neuroprotection in various animal models of neurodegenerative diseases. We found that following induction, the mouse stromal cells demonstrate neuronal phenotype and express the neuronal marker, NeuN (neural nuclei protein). However, most of the stromal cells derived from the Tg mice, but not the wild type, also expressed human Bcl-2, as indicated by immunocytochemistry. Furthermore, these induced neuron-like cells were more resistant to cell death induced by dopamine. In conclusion, our experimental models showed that stromal cells might be induced to neuronal phenotypes and activate neuronal-specific promoters. Moreover, neurons targeted over expression of the human *bcl-2* gene and provided high resistance against such apoptotic insults. This novel strategy reveals a new horizon in the improvement of gene therapy, based on stem cell transplantation in neurodegenerative diseases.

Index Entries: Bone marrow stromal cells; Bcl-2; neuronal plasticity; neuron-specific enolase promoter.

Background

Specific populations of neurons are destroyed in certain neurodegenerative diseases (e.g., dopaminergic neurons of the substantia nigra in patients with Parkinson's disease, lower motor neurons in amyotrophic lateral sclerosis). Treatment for these diseases, if available, is mainly symptomatic. Replacements of the degenerated neurons by potent fetal or stem cells have already been used in animal models and in Parkinsonian patients, but results have proved inconclusive (Freed et al., 2001; Kim et al., 2002).

In recent years, there has been an increasing interest in adult bone marrow-derived stromal stem cells that support hematopoiesis. These mesenchymal stem cells differentiate into connective tissue, muscle, bone, cartilage, and fat cells (Prockop, 1997; Pittenger et al., 1999; Deans and Moseley, 2000). Evidence has accumulated that human, rat, and mouse bone marrow stromal (mBMS) cells can also be induced to differentiate to neuron-like cells in cultures

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(Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002; Black and Woodbury, 2001; Deng et al., 2001; Kohyama et al., 2001; Reyes and Verfaillie, 2001; Jiang et al., 2002). Following induction, most (up to 80%) stromal cells, may exhibit neuronal phenotypes (Woodbury et al., 2000, 2002). Moreover, it was shown that the differentiated cells express neuronal protein markers such as neuron-specific enolase (NSE), neural nuclei protein (NeuN), neurofilament-M (NF-M), and trkA. Other experiments with rodents demonstrated that transplanted bone marrow-derived cells might migrate into various brain regions and develop neuron-like features (Brazelton et al., 2000; Mezey et al., 2000; Chen et al., 2001; Li et al., 2001; Lu et al., 2001a,b, 2002; Hess et al., 2002; Li et al., 2002; Zhao et al., 2002). Furthermore, Mezey et al. (2003) found Y-chromosomes in the human brains of females following transplantation of male bone marrow. Donor cells were found in several selective brain regions, especially in the hippocampus and cerebral cortex. However, other researchers claim that bone-to-brain transdifferentiation might not be a general phenomenon but might reflect fusion with neurons or transient expression of many proteins, including neuronal markers (Holden and Vogel, 2002; Lemischka, 2002; Wurmser and Gage, 2002).

To exclude these possibilities, bone marrow from adult transgenic (Tg) mice that express cellular markers as green fluorescent protein was used for transplantation. In these experiments bone marrow-derived cells injected into the brain or even the tail vein migrated into several regions of the brain, including the olfactory bulb, the cortex, the hippocampus, and the cerebellum (Brazelton et al., 2000; Mahmood et al., 2001). Some of the marrow-derived neuronal cells also grew long fibers and produced a protein indicating neuronal activity. These findings suggest that the marrow-derived neurons not only entered the brain but also survived and functioned.

A major limitation of brain cellular transplantation of fetal or stem cell-derived neurons is that few grafted cells (3–20%) survive the procedure (Brundin et al., 2000). It was found that the initiation and first few days of implantation are crucial for the survival of the donor cells, most of which undergo apoptosis triggered by hypoxia, hypoglycemia, mechanical trauma, free radicals, growth factor deprivation, and excessive extarcellular concentrations of excitatory amino acid in the host brain (Brundin et al., 2000). Indeed, inhibition of apoptosis by treatment with caspase inhibitors demonstrated significant positive effects on the survival of the transplanted cells (Brundin et al., 2000; Cicchetti et al., 2002).

In our previous studies, we found that cultured neurons overexpressing the antiapoptotic gene *bcl-2* are protected against apoptosis induced by a series of neurotoxins (Offen et al., 1997; Ziv et al., 1997). The *bcl-2* family plays a central role in the mitochondrial integrity and caspase activation, and therefore in the apoptotic process (Adams and Cory, 1998; Pettmann and Henderson, 1998). Previous experiments with Tg mice expressing human Bcl-2 under neuron-specific enolase promoter (NSE-hBcl-2) demonstrated low susceptibility to neuronal death during development and throughout adult life (Farlie et al., 1995; Bernard et al., 1997) and high resistance in several models of neurodegeneration (Offen et al., 1998, 2000).

Based on these findings, we used induced bone marrow stromal cells derived from NSE-hBcl-2 mice to initiate differentiation into neuron-like cells expressing a high level of human Bcl-2 and NeuN protein. We found that differentiated NSE-hBcl-2 neuron-like cells are resistant to dopamine-induced cell death. The potential usefulness of antiapoptotic gene induction in stem cell based treatment of neurological disease is discussed.

Materials and Methods

Animals

Primary cultured mBMS cells were obtained from adult C57/bl or heterozygous Tg mice bearing the human *bcl-2* gene under control of the NSE-hBcl-2 promoter. The mice were generated and kindly provided by Ora Bernard, Melbourne Australia (Farlie et al., 1995). Tail DNAs were prepared and analyzed by PCR to identify their genotype as described previously (Offen et al., 2000).

Isolation of MBMS Cells

Mice were sacrificed by cervical dislocation and prepared with 70% alcohol solution. After tibias and femurs were removed and placed in Hank's balanced salt solution (HBSS; Biological Industries, Bet-Haemek, Israel), mBMS cells were collected by flushing out the marrow using a syringe (1 mL) with a 25-gage needle, filled with 0.5 mL sterile HBSS. Cells were disaggregated by gentle pipetting several times until a milky homogenous single-cell suspension was achieved. Bone marrow aspirate was

diluted and washed by adding 5 mL HBSS, centrifuged at 1000*g*, for 20 min at room temperature (RT), and removing the supernatant. The cell pellet was resuspended in 1 mL growth medium and diluted to 10 mL. The cells were plated in polystyrene plastic tissue-culture 75-cm^2 flasks (Corning, Corning, NY).

Cell Culture

Mouse bone marrow stromal cells were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM; Biological Industries) supplemented with 15% fetal calf serum (FCS; Biological Industries), 5% horse serum (Biological Industries), 1× nonessential amino acid (Biological Industries), 0.001% β-mercaptoethanol (Sigma, St. Louis, MO), 2 m*M* glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, and 12.5 U/mL nystatin (SPN; Biological Industries), maintained at 37°C in a humidified 5% CO_2 incubator. After 48 h of incubation, the nonadherent layer was removed. The tightly adhered mBMS cells were washed twice with Dulbecco's phosphate-buffered saline (PBS; Biological Industries), and fresh growth medium was added. The medium was replaced every 3 or 4 d, and when cells reached 70–90% confluency, cultures were harvested with trypsin-EDTA solution (0.25% trypsin and EDTA 1:2000 in Puck's saline; Biological Industries) for 5 min at 37°C. About 10 mL of growth medium was mixed with the trypsinized cells to inactivate the trypsin. The cells were removed and used immediately or replated (up to eight times).

Neural Differentiation

Seventy-two hours prior to differentiation, growth medium was replaced with predifferentiated medium consisting of DMEM supplemented with 10% FCS, 2 m*M* glutamine, SPN, 10 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN), 10 ng/mL epidermal growth factor (EGF; R&D Systems), and N2 supplement (5 µg/mL insulin; 20 n*M* progesterone; 100 µ*M* putrescine; 30 n*M* selenium; 100 µg/mL transferrin [Bottenstein, 1985]). To induce differentiation to neuron-like cells, the predifferentiated media was removed and cells were washed with PBS and transferred to the differentiated medium, composed of DMEM supplement with 2 m*M* glutamine, SPN, N2 supplement, 200 µ*M* butylated hydroxyanisole (BHA; Sigma), 1 m*M* dibutyryl cyclic AMP (dbcAMP; Sigma), 0.5 m*M* 3-isobutyl-1-methyl-xanthine (IBMX; Sigma), and 10 µ*M* all-*trans*-retinoic acid (RA; Sigma) for 6–72 h. The differentiated mBMS cells were further analyzed as described below.

Western Blot Analysis

Protein Preparation from Mouse Brain

Brains obtained from adult C57/bl or heterozygous NSE-hBcl-2 Tg mice, were gently homogenized with a glass-glass homogenizer in 5 vol (w/v) of cold 100 m*M* PBS (pH 7.0) containing protease inhibitor cocktail tablets, Complete™ (Boehringer Mannheim GmbH, Mannheim, Germany). Homogenates were centrifuged at 1000*g* for 10 min at 4°C, and supernatants were collected. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL).

Protein Preparation from Cell Culture

After appropriate treatment, wild-type (WT) and Tg mBMS cells (1×10^6) were washed with PBS, scraped, and pelleted by centrifugation at 1000*g* for 10 min. The pellet was resuspended in 50 µL of cold buffer containing 105 m*M* Tris (Sigma), 5 m*M* EDTA (BDH Laboratory Supplies, Poole, England), 140 m*M* NaCl (BioLab, Jerusalem, Israel), 10 m*M* sodium fluoride (Sigma), 0.5% NP-40 (U.S. Biochemical Corporation, Cleveland, OH), and 1 µ*M* phenylmethylsulfonyl fluoride (Sigma). Cells were then incubated on ice for 30 min and vortexed for 10 s at high speed every 5 min. The mixture was centrifuged at 13,000*g* for 20 min at 4°C, and supernatants were collected. Protein content was determined by the BCA protein assay kit (Pierce).

Protein Separation and Immunostaining

Eighty micrograms of protein extract was denatured in a sample buffer (62.5 m*M* Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% 2-β-mercaptoethanol, 0.0025% bromophenol blue [Sigma]), diluted 1:5 with the sample and boiled for 5 min. Each sample was loaded on a 12.5% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories), followed by blocking with 5% nonfat milk in Tris-buffered saline (TBS: 10 m*M* Tris at pH 7.5, 150 m*M* NaCl) with 0.1% Tween-20 (blocking solution). The membranes were probed overnight, at 4°C, with rabbit anti-human Bcl-2 (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-NeuN (1:1000 dilu-

tion, Chemicon, Temecula, CA), or mouse antiactin (1:1000 dilution, Chemicon) in 0.375% nonfat milk in TBS-T. The next day, membranes were washed twice (15 min each) with blocking solution and once with TBS-T for 15 min. Membranes were then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG diluted at 1:25,000 or anti-mouse IgG diluted at 1:20,000 (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.375% nonfat milk in TBS-T for 30 min at RT. The membranes were then washed twice (15 min each) with blocking solution and once in TBS-T for 15 min and were stained using the enhanced SuperSignal® chemiluminescent detection kit (Pierce) and exposed to medical X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometry of the specific proteins bands was preformed by VersaDoc® imaging system (Bio-Rad Laboratories) and Quantity One® software (Bio-Rad).

Immunocytochemistry

Wild-type and Tg mBMS cells were plated and treated in slide chambers (Nalge Nunc International. Napervilee, IL) previously treated aseptically with poly-L-lysine, molecular weight (mol wt) 70,000–150,000 (Sigma). The cells were fixed with 4% paraformaldehyde in PBS (pH 7.3) for 30 min at 4°C and 30 min at RT. The slides were then washed three times with PBS (5 min each) and permeabilized with PBS containing 0.1% Triton X-100 (Sigma) and 10% goat serum (to block nonspecific binding sites [Biological Industries]) for 10 min at 4°C and 10 min at RT. The slides were then washed three times with PBS (5 min each). The endogenous peroxidase was blocked by adding 3% H_2O_2 (Merck, Dramstadt, Germany) in methanol absolute (Bio-Lab, Jerusalem, Israel) for 20 min at RT. Following three washes with PBS (5 min each), slides chambers were incubated overnight at 4°C with rabbit anti-human Bcl-2 diluted at 1:20 (Calbiochem, San-Diego, CA) or mouse anti-NeuN diluted at 1:40 (Chemicon); primary antibodies were diluted in PBS containing 10% goat serum. The next day the slides were washed thoroughly three times in PBS (10 min each). Biotin-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) antibodies at 7.5 µg/mL, diluted in 10% goat serum and 0.2% Tween-20 in PBS, were applied for 30 min at RT. After washing three times with PBS (5 min each), the slides were incubated for 30 min at RT in avidin conjugated to horseradish peroxidase (Vectastain® ABC kit, Vector Laboratories). The slides were washed three times with PBS and incubated in peroxidase substrate solution (3,3'-diaminobenzidine tetrahydrochloride and H_2O_2; Zymed Laboratories, San Francisco, CA) until the desired stain intensity was developed. After the reaction was stopped by water, coverslips were mounted on slides with glycerol vinyl alcohol mounting solution (Zymed Laboratories), and slides were examined with a light microscope (*see* below).

Quantification of Cell Survival

Bone marrow stromal cells from Tg and WT mice (~2×10^5 cells/mL) were seeded in poly-L-lysine (mol wt 70,000–150,000)-coated 96-well multidishes (Corning Incorporated) in 100 µL growth medium over night and induced to differentiate for 24 h, as described above. After 6 h of incubation, 10–500 µ*M* of dopamine (Research Biochemicals International, Natick, MA) was added. Each treatment was repeated four times. To assay survival, cells were incubated for 10 min at RT with 10 µg/mL Hoechst 33342 dye (Sigma) and 10 µg/mL propidium iodide (PI [Sigma]) diluted in PBS. Hoechst 33342 stained all nuclei, whereas PI stained only those of cells with a disrupted plasma membrane. Cell morphology was examined as follows: (1) Viable cells had blue-stained normal smooth nuclei; (2) nonviable, necrotic cells had normal sized red-stained smooth nuclei; (3) apoptotic cells had red-stained nuclei, and (4) the chromatin was either fragmented multiple bright specks or condensed with one or more spheres. Pictures were taken at random from four fields after each treatment. The blue- and red-stained nuclei were counted by Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD). Results are presented as mean ± S.E.M.; p values were calculated using two-tailed unpaired Student's t-test. In all tests, significance was assigned when $p < 0.05$.

Microscopes

Photographs of differentiated and nondifferentiated mBMS cells were taken with a light Olympus IX70-S8F2 microscope (Olympus, Tokyo, Japan). All slides of immunocytochemistry were analyzed under an Olympus BX52TF microscope. Hoechst 33342- and PI-stained cells were examined with a fluorescence Olympus IX70-S8F2 microscopy with fluorescent light source (excitation wavelength, 330–385 nm; barrier filter, 420 nm) and a U-MNU filter cube (Olympus). The DP50 microscope digital camera system (Olympus) was used to capture the pictures. Images from the microscope were acquired using adigital camera and ViewfinderLite™ software

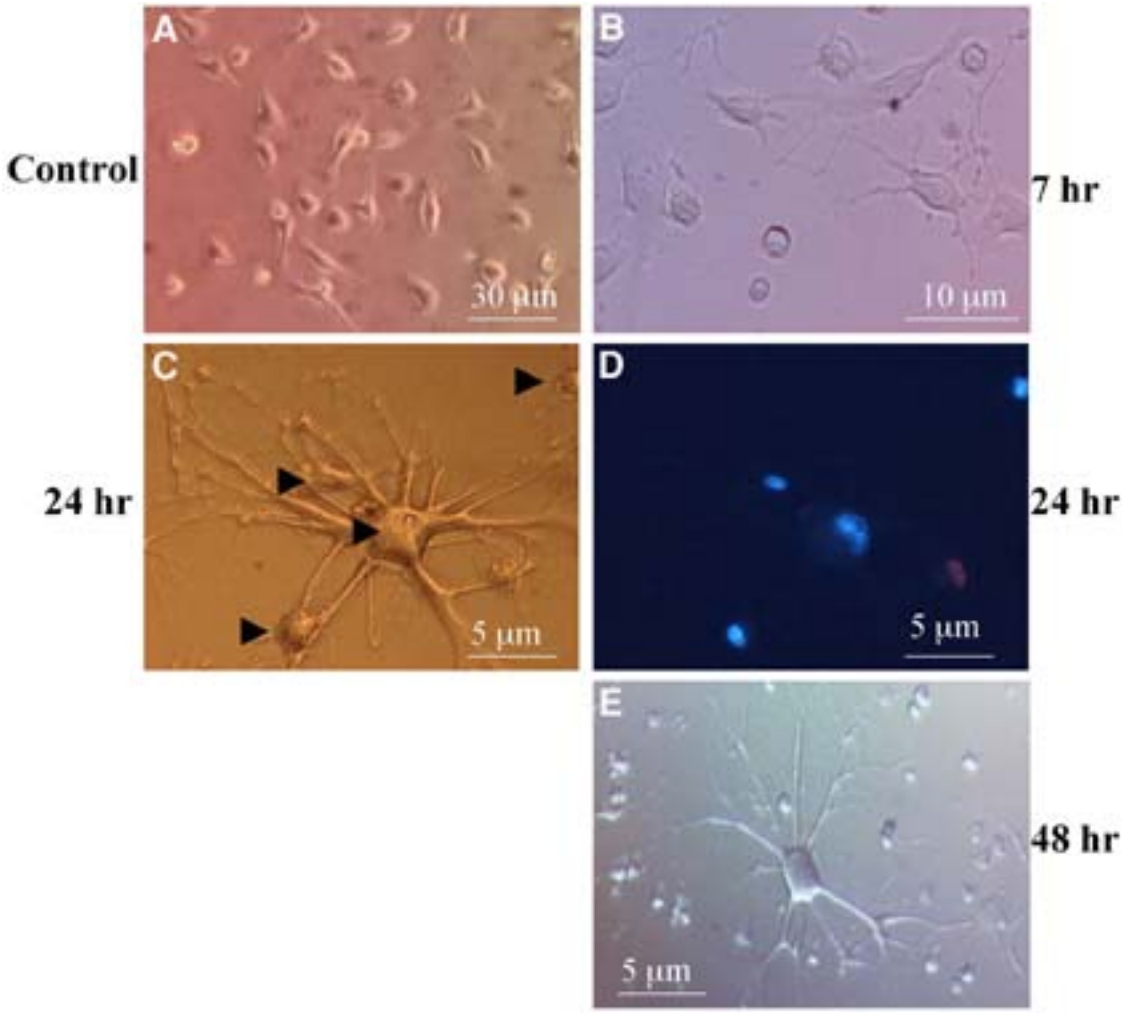

Fig. 1. Adult Tg mBMS cells differentiated into neurons. Transgenic mBMS cells were incubated with growth medium **(A)** or differentiation medium for 7 h **(B)**, or 24 h **(C)**, or 48 h **(E)**. Cells following 24-h differentiation were stained with Hoechest 33342; the viable cells are seen in **D** and indicated by arrowheads in **C**.

(Olympus). StudioLite™ software (Olympus) was used to edit and analyze the filmed images.

Results

Induction of mBMS Cells to Differentiate into Neuron-Like Cells

Induction of mBMS cells with differentiation medium containing BHA, RA, N2 supplement, and elevated cAMP resulted in significant morphological changes (Fig. 1B,C). Within 1 h of exposure, the cytoplasm in the adherent mBMS cells condensed and retracted toward the nucleus, creating a spherical structure; in a few cells, branches around the cells started to develop. This phenomenon was repeated during the next few hours, and more cells demonstrated a neuron-like appearance. Seven hours postdifferentiation, cell bodies that became increasingly spherical and refractile exhibited a typical neuronal structure (Fig. 1B). After 24 h of treatment, cells were transformed into spindle-shaped, elaborately long branching processes with growth cone-like terminal structures that frequently made contact with other mBMS cells, thereby exhibiting a typical neuronal presentation (Fig. 1C). Most of the cells died after 96 h of differentiation.

Differentiated mBMS Cells from Tg Mice Expressing NeuN and Human Bcl-2

Bcl-2 and NeuN protein expression was examined in brain extracts from Tg and WT mice. Western blot analysis confirmed expression of NeuN in both mice

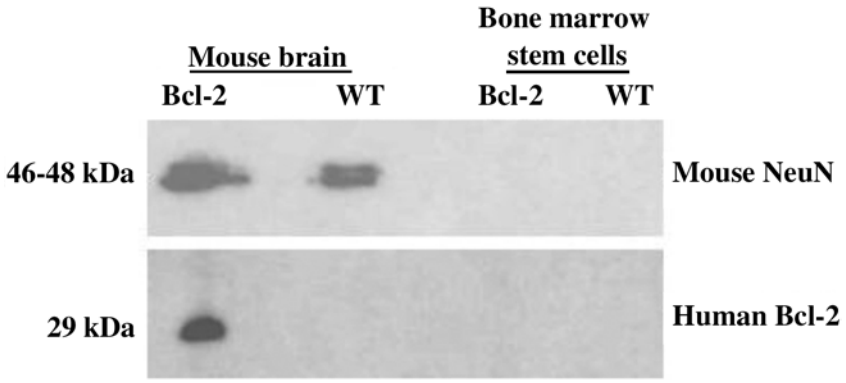

Fig. 2. Bcl-2 and NeuN expression in extracts from brains and mBMS cells of WT and human Bcl-2 Tg mice. Western blot analysis confirms expression of Bcl-2 only in Tg mouse brain extract and is negative in WT mouse brain extract. The expression of Bcl-2 is negative in both WT and Tg mBMS cells. NeuN was expressed in brain extract from WT and Tg mice but not in the mBMS cells.

strains. However, human Bcl-2 was expressed only in the Tg mouse brain extract (Fig. 2).

Extracts of undifferentiated mBMS cells from WT and Tg mice did not express either human Bcl-2 or NeuN (Fig. 2).

To verify whether differentiated mBMS cells activate neuronal promoters and express neuronal markers, we followed the expression of human Bcl-2 and NeuN using specific antibodies. Mouse bone marrow stromal cells were induced for differentiation by 24-h cell exposure to predifferentiation medium, followed by incubation for 6–48 h with differentiation medium. Cells were then fixed on slides and stained with antibodies against NeuN and human (but not mouse) Bcl-2. As seen in Figs. 3 and 4, undifferentiated cells isolated from WT or Tg mice were negative to human Bcl-2 and NeuN staining. In contrast, mBMS cells derived from Tg mice demonstrated NeuN and human Bcl-2 expression 6 h after induction (Figs. 3 and 4). Interestingly, we found a positive correlation between the advance in neuronal morphology and the intensify of the NeuN signal.

Using Western blot assays, we found significant increase in the expression of both human Bcl-2 and NeuN after incubation for 24–72 h in the differentiation medium (Fig. 5). It was interesting to note that low levels of human Bcl-2 and NeuN could be detected in mBMS cells even before induction, indicating the potential of cultured mBMS cells to differentiate in vitro.

Differentiated mBMS Cells Are Resistant to Dopamine Toxicity

As the differentiated mBMS cells overexpress the antiapoptotic gene, human *bcl-2*, we examined whether these cells showed increased resistance to an apoptotic-induced neurotoxin. Cells from WT and Tg mice, differentiated for 6 h, were also exposed to various concentrations of dopamine (10–500 μ*M*) for an additional 18 h. Cell survival was analyzed by fluorescence microscopy following staining with Hoechst 33342 and PI nuclear dyes. Cells that lost their plasma membrane permeability barrier were stained by (red) PI, whereas viable nuclei DNA were marked by the blue Hoechst 33342 dye (Lee et al., 1997).

Using this double staining method we found that >80% of the WT differentiated mBMS cells were nonviable as compared to 24% of the differentiated cells derived from the Tg mice after 18 h of treatment with 200 μ*M* dopamine (Fig. 6). The difference in the resistance to dopamine was demonstrated in the exposure between 10 and 200 μ*M*, whereas 500 μ*M* mBMS cells from both mice strains revealed similar toxicity.

Discussion

Our study demonstrated that bone marrow stromal cells (BMSCs) might change their designations by induced differentiation in culture. The differentiation of mBMS cells into neuron-like cells was evident in the dramatic morphological changes observed. Before treatment, mBMS cells displayed a flat, fibroblastic morphology, whereas at 24 h posttreatment the cells were rounded, exhibited highly retractile cell bodies, and displayed prominent process-like extensions. Morphological changes were accompanied by the expression of the tissue-specific neuronal marker, NeuN. Moreover, BMSCs isolated from the Tg mouse showed that prior to the induction of differentiation, they expressed a very

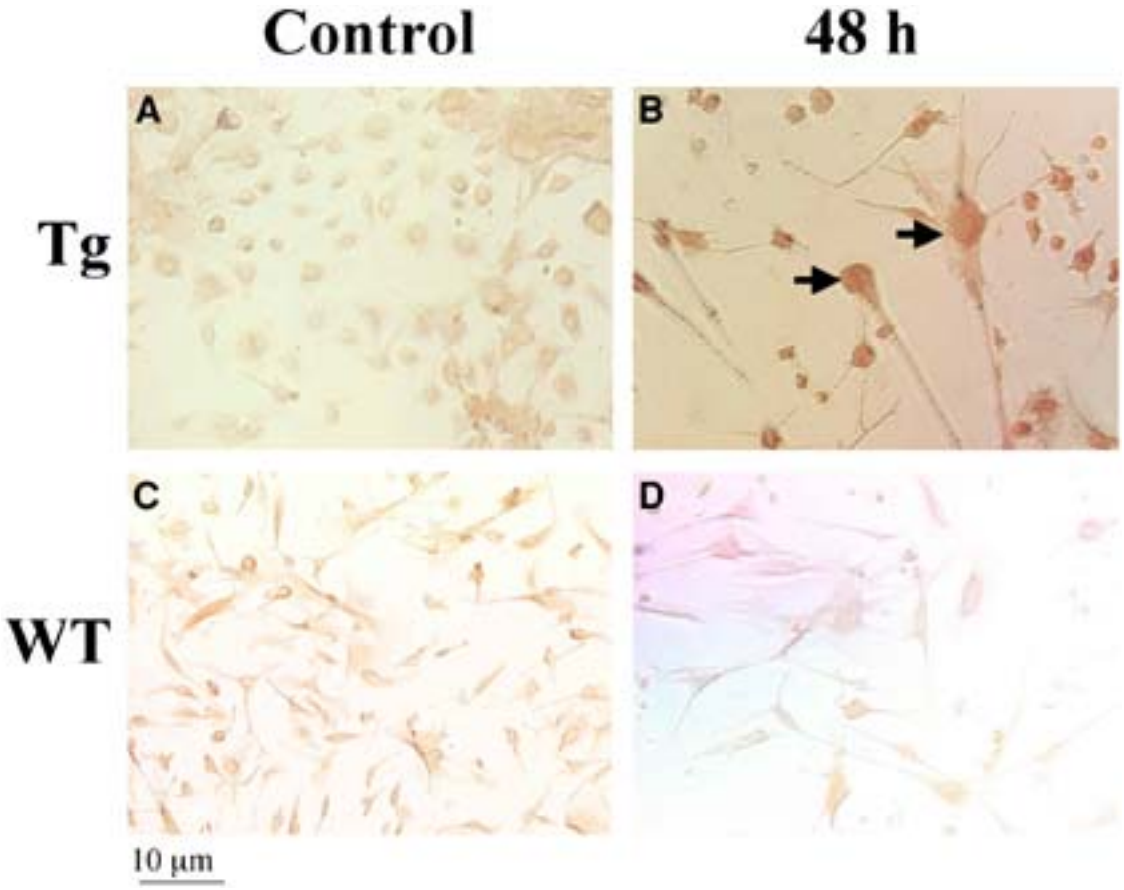

Fig. 3. Human Bcl-2 is expressed only in differentiating adult transgenic mBMS cells. mBMS cells from WT and Tg mice, following 48 h of differentiation, were analyzed by immunocytochemistry for human Bcl-2 expression. Undifferentiated Tg mBMS cells **(A)** did not stain with antihuman Bcl-2. **(B)** Intense human Bcl-2 staining is evident in mBMS cells incubated with differentiation medium for 48 h. Undifferentiated **(C)** and differentiated **(D)** cells isolated from WT mice were negative to human Bcl-2 staining.

low level of human Bcl-2, whereas treatment with differentiation medium activated the NSE promoter downstream of the gene, which induced significant increase in expression. Overexpression of *bcl-2* in the differentiated Tg mBMS cells provided protection against dopamine toxicity, compared to WT cells, as measured by PI and Hoechest 33342 staining.

Bone marrow stromal cells provide the structural and functional support for the generation of blood cell lineages from hematopoietic stem cells. They consist of morphologically and biochemically distinct cell types: bone marrow fibroblast-reticular cells, adipocytes, osteoblasts, macrophages, and endothelial cells. Bone marrow stromal cells can be cultivated and contain progenitors capable of generating bone, cartilage, fat, and other connective tissues. These nonhematopoietic precursors found in BMSCs are also known as colony-forming-unit fibroblasts and mesenchymal stem cells (Prockop, 1997). It was demonstrated that under specific experimental conditions, BMSCs can also be induced to differentiate to skeletal (Ferrari et al., 1998), cardiac muscle (Makino et al., 1999), and hepatocyte (Petersen et al., 1999) cells that are not part of their normal repertoire. Our present study, along with those published by others, indicates that BMSCs retain the capacity to differentiate into neuron-like cells. It is worth noting that BMSCs are mesodermal-derived tissue, whereas neurons are classical ectodermal tissue. The changes in morphological features, such as retractile cell bodies and long branching processes, seen in our experiments, are similar to those demonstrated induction of differentiation of human, murine, and rat BMSCs

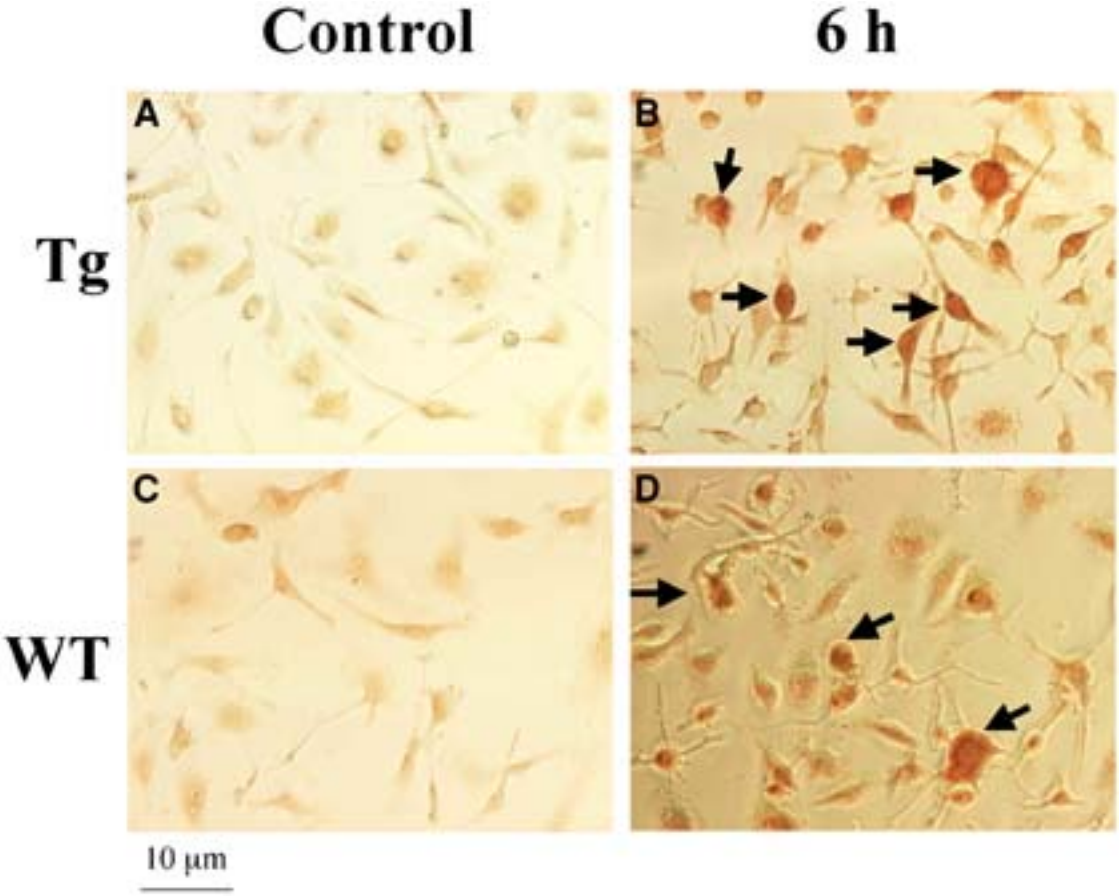

Fig. 4. NeuN is expressed in differentiated adult mBMS cells. Undifferentiated mBMS cells from Tg **(A)** and WT **(C)** mice did not stain for NeuN protein. Intense NeuN staining is evident in both Tg **(B)** and WT **(D)** mBMS cells after 6 h of differentiation.

(Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002; Deng et al., 2001; Kohyama et al., 2001). Moreover, it was reported that rat and human BMSCs that differentiated to neuron-like cells exhibited increased characteristics of neuronal protein markers such as β-tubulin III, NeuN, NF-M, NSE, tau, and trk-A (Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002; Deng et al., 2001). In addition, the induced culture demonstrated several astrocyte and oligodendrocyte markers such as glial fibrillary acidic protein (GFAP), vimentin, myelin-basic protein, and galactocerebroside (Gal-C) (Reyes and Verfaillie, 2001). Characteristic neuronal markers are summarized in table 1. In addition, it was demonstrated that neuron-like cells generated from marrow stroma-formed neuritis began to respond to depolarizing stimuli and decreasing resting membrane potential similar to mature functional neurons (Kohyama et al., 2001).

In our experiments, BMSCs were isolated by culturing the cells on plastic dishes and continuing only with the adherent cells. In this way, most of the hematopoietic cells and progenitors were depleted. For proliferation, cells were maintained with growth medium containing EGF, as described by Sanchez-Ramos et al. (2000), and bFGF shown to stimulate cell proliferation of mesodermal tissues. In addition, bFGF was shown to induce neuron differentiation, survival, and regeneration (Gremo and Prestab, 2000). We also added the N2 supplement to provide improved conditions for neural expansion (Bottenstein, 1985). To induce differentiation, we cultured mBMS cells under conditions that increased intracellular cAMP by using IBMX, a phosphodiesterase inhibitor, and cAMP analog, dbcAMP. It was demonstrated that IBMX or dbcAMP could greatly increase the extension of processes in a medulloblastoma cell

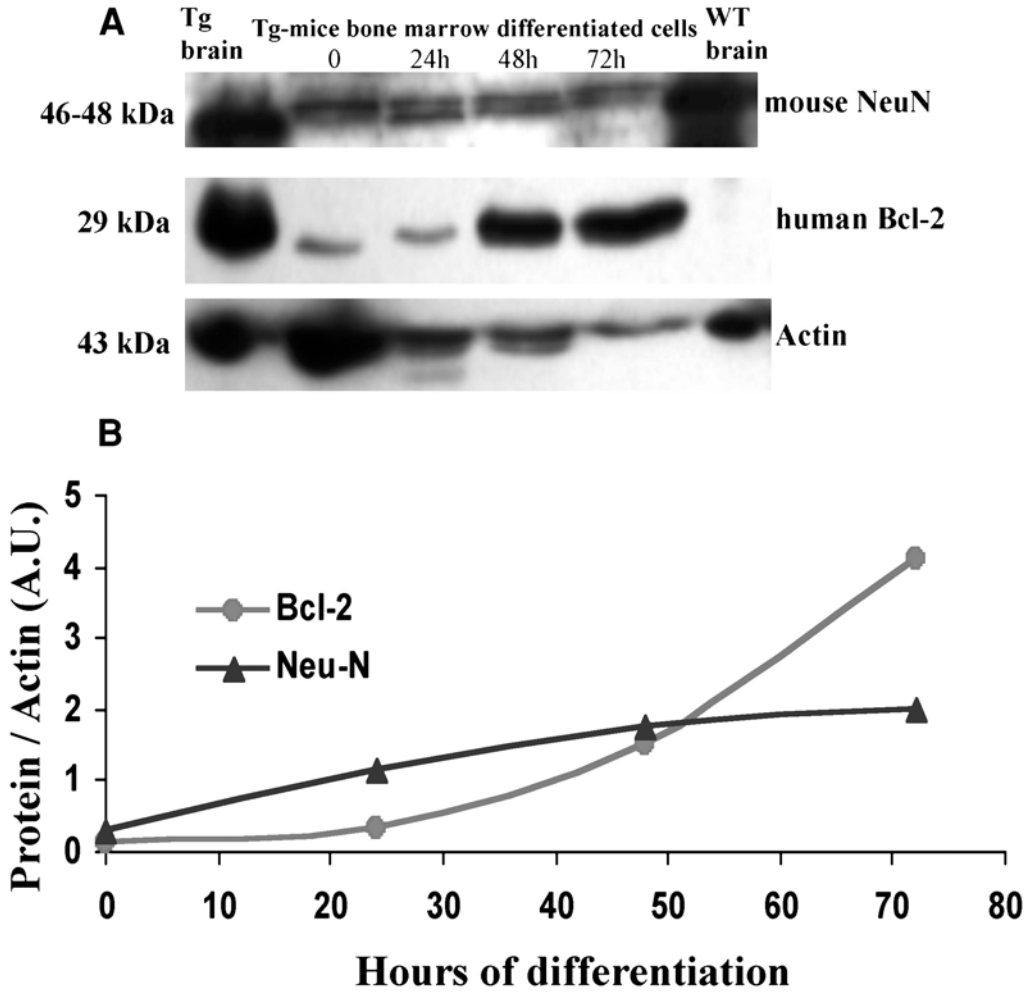

Fig. 5. Elevation of expression of NeuN and human Bcl-2 by differentiating human Bcl-2 Tg mBMS. **(A)** Western blot analysis of noninduced mBMS cells demonstrated low levels of human Bcl-2 and NeuN expression. Expression was markedly increased 72 h after induction of differentiation. Expression of NeuN was seen in both brain extracts. However, human Bcl-2 was expressed only in the Tg brain extract. **(B)** Densitometry of NeuN and human Bcl-2 protein bands compared to actin.

line, MCD-1 (Moore et al., 1996). The formation of long processes induced by IBMX was associated with a decrease in cell proliferation. Moreover, Deng et al. (2001) found that elevation of cAMP through addition of dbcAMP and IBMX induced a neuronal morphology in human BMSCs. The mechanism by which BHA induces neuronal differentiation is unclear. However, its antioxidant properties, which enhance neuronal survival in vitro, might be partially responsible for the present neuronal induction (Woodbury et al., 2000). RA, the vitamin A metabolite, is involved in three fundamental aspects of the development of the central nervous system (CNS): (1) the stimulation of axon outgrowth, particularly neuronal subtypes; (2) the migration of the neural crest; and (3) the specification of rostrocaudal position in the developing CNS in the forebrain, midbrain, hindbrain, and spinal cord (Maden and Holder, 1992). RA also controls cellular differentiation and cellular proliferation in the normal and transformed neuron. Moreover, RA can indirectly modulate differentiation of neurons by modifying the expression of neuronal cell-surface receptors to peptide growth factors (Scheibe and Wagner, 1992).

The fact that BMSCs can give rise to neural cells is not surprising, as nestin, the marker for the neural precursors, is expressed in BMSCs (Sanchez-Ramos et al., 2000). Moreover, in the present study, we

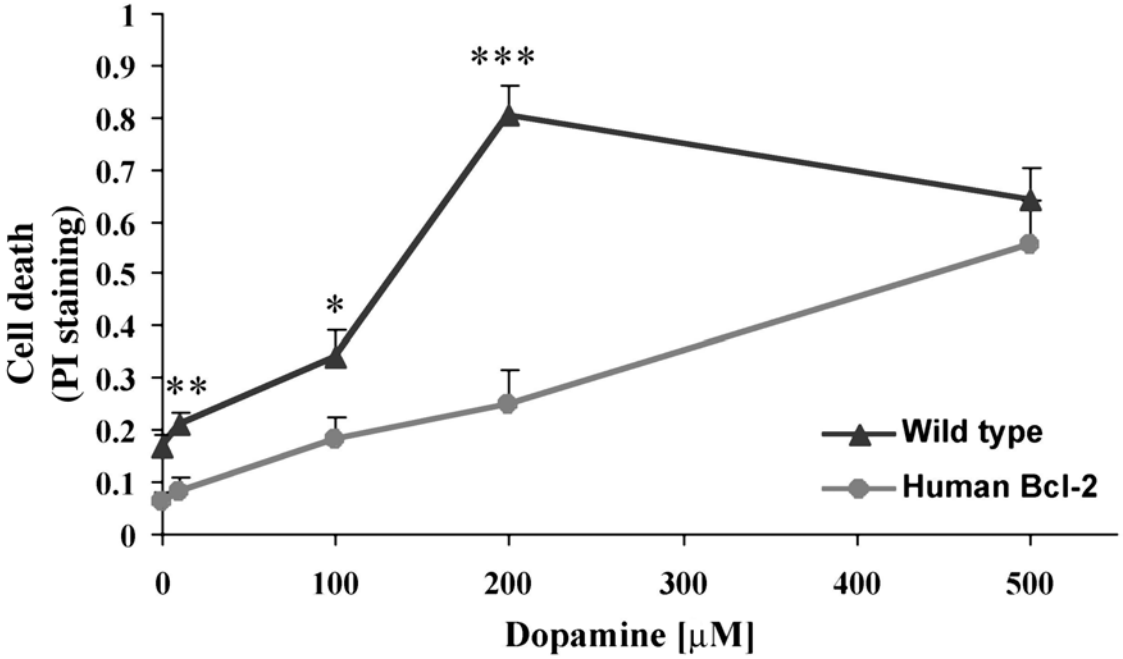

Fig. 6. Differentiated stromal cells derived from human Bcl-2 Tg mice are more resistant to dopamine toxicity than those from WT mice. Differentiated mBMS cells were incubated for 18 h with elevated concentrations of dopamine. Transgenic mBMS cells were much more resistant to dopamine toxicity, as measured by PI staining. (Means ± S.E.M., $n = 4$, Student's t-test, $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$).

Table 1
Protein Markers in Bone Marrow Stromal Cells Differentiated into Neuron-Like Cells

Source of cells	Markers of neural lineage	Markers of astrocyte lineage	Markers of oligodentrocyte lineage	References
Human	[4]β-tubulin III, [1]NeuN; [2]NF-M, [2–4]NSE, [1]nestin	[1,4]GFAP, [3]vimentin	[4]Myelin-basic protein, [4]Gal-C	[1]Sanchez-Ramos et al. (2000); [2]Woodbury et al., (2000); [3]Deng et al., (2001); [4]Reyes and Verfaillie (2001)
Rat	[1]NSE, [1]NF-M, [1]NeuN, [1,2]tau, [1]trk-A, [2]β-tubulin III, [2]synaptophysin, [1]nestin			Woodbury et al., ([1]2000, [2]2002)
Mouse	[1–3]NeuN, [2]β-tubulin III, [2]Hu, [1,2]microtubule-associated protein 2 (MAP2)	[2]GFAP	[2]Gal-C	[1]Sanchez-Ramos et al. (2000); [2]Kohyama et al. (2001); [3]this article

demonstrated that low levels of human Bcl-2 and NeuN are expressed even in the absence of differentiation factors.

By using the transgenic mouse model for the expression of the human *bcl-2* gene, we achieved two goals. First, we demonstrated that our method of inducing mBMS cells to neurons activates the specific promoters of neuronal tissue. Second, the *bcl-2* gene expressed in neuronal tissue provided protection against apoptosis-induced cell death. Indeed, several studies, including ours, demonstrated in several animal models that manipulations in the *bcl-2*

or *bax* genes affect the neurodegeneration process (Kostic et al., 1997; Offen et al., 1997, 1998, 2000; Hochman et al., 1998; Vila et al., 2001). To our knowledge, this is the first report that demonstrates targeted expression of a transgene in neuron-like cells derived from BMSCs.

In conclusion, our observations, along with those published by others, suggest that adult BMSCs have a potentially larger developmental repertoire than previously appreciated. The data suggest that intrinsic genomic mechanisms of commitment and lineage restriction are plastic. Bone marrow stromal cells may be used for cell therapy and replace neurons in degenerative diseases. Furthermore, differentiated BMSCs might express transgenes that provide protection against cell death occurring during brain transplantation.

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