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In Vitro Differentiation of Human Marrow Stromal Cells into Early Progenitors of Neural Cells by Conditions That Increase Intracellular Cyclic AMP

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Human marrow stromal cells (hMSCs) are multipotential stem cells that can be differentiated into bone, cartilage, fat, and muscle. In the experiments here, we found that undifferentiated cultures of hMSCs express some markers characteristic of neural cells such as microtubule-associated protein 1B (MAP1B), neuron-specific tubulin (TuJ-1), neuron-specific enolase (NSE), and vimentin. By treating hMSCs with 0.5 mM isobutylmethylxanthine (IBMX)/1 mM dibutyryl cyclic AMP (dbcAMP) for 6 days, about 25% of the hMSCs differentiated into cells with a typical neural cell morphology and with increased levels of both NSE and vimentin. The data suggested that the hMSCs may have been differentiated into early progenitors of neural cells *in vitro* under conditions that increase the intracellular level of cAMP. © 2001 Academic Press

Key Words: **marrow stromal cells; differentiation; neural cells; IBMX; cyclic AMP.**

Human marrow stromal cells (hMSCs) are multipotential adult stem cells that contribute to the regeneration of tissues such as bone, cartilage, fat, and muscle (1–4). Recent data suggest that MSCs can also be induced to differentiate into neural cells *in vivo.* Azizi *et al.* (5) found that hMSCs integrated and migrated along the known pathway for the migration of neural stem cells after being infused into rat brain. Kopen *et al.* (6) injected mouse marrow stromal cells (mMSCs) labeled with BrdU into lateral ventricle of a 3-day-old neonatal mouse. Twelve days later, the mMSCs had migrated to both the forebrain and the cerebellum without disruption of the host normal brain structure. Some of the mMSCs differentiated into cells that had the morphology of astrocytes and that expressed the astrocyte-specific protein glial fibrillary acid protein (GFAP). Some of the cells appeared in neuron-rich regions such as the olfactory bulb and the internal granular layer of the cerebellum. Furthermore, some BrdU-labeled cells within the reticular formation of the brain stem appeared to be positive for neurofilament, suggesting that the mMSCs differentiated into neurons.

Recently, two reports described conditions under which MSCs can be differentiated in culture to neural-like cells. In one report (7), the cells were differentiated either by serum withdrawal and exposure to beta-mercaptoethanol (BME), or by treatment with butylated hydroxytoluene (BHT) and DMSO. In a second report (8), the cells were differentiated either by treatment with EGF followed by BDGF, or by coculture with suspensions of rat or mouse midbrain cells. Here we describe similar but not identical differentiation of MSCs to cells with some but not all of the characteristics of early neurons and glia by treatment with dibutyryl cyclic AMP (dbcAMP) and isobutylmethylxanthine (IBMX), conditions that increase intracellular cyclic AMP.

MATERIALS AND METHODS

Isolation and culture of hMSCs. Twenty-milliliters bone marrow aspirates were taken from the iliac crest of normal donors ranging in age from 19 to 49 years old. Isolation and culture of hMSCs were carried out as previously described by DiGirolamo *et al.* (9). Briefly, the 20 ml aspirate was diluted 1:1 with Hanks' balanced salt solution (HBSS, GIBCO BRL) and layered over 10 ml Ficoll (Ficoll–Paque, Pharmacia). After centrifugation at 2500*g* for 30 min, the mononuclear cell layer was recovered from the gradient interface and washed with HBSS. The cells were centrifuged at 1500*g* for 15 min and resuspended in complete culture medium (α-MEM, GIBCO BRL; 20% fetal bovine serum (FBS) lot—selected for rapid growth of hMSCs, Atlanta Biologicals; 100 units/ml penicillin; 100 μg/ml streptomycin; and 2 mM L-glutamine, GIBCO BRL). All of the cells were plated in 25 ml medium in a 150-cm^2 culture dish (Falcon) and incubated at 37°C with 5% humidified CO_2. After 24 h, nonadherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline. Fresh complete culture me-

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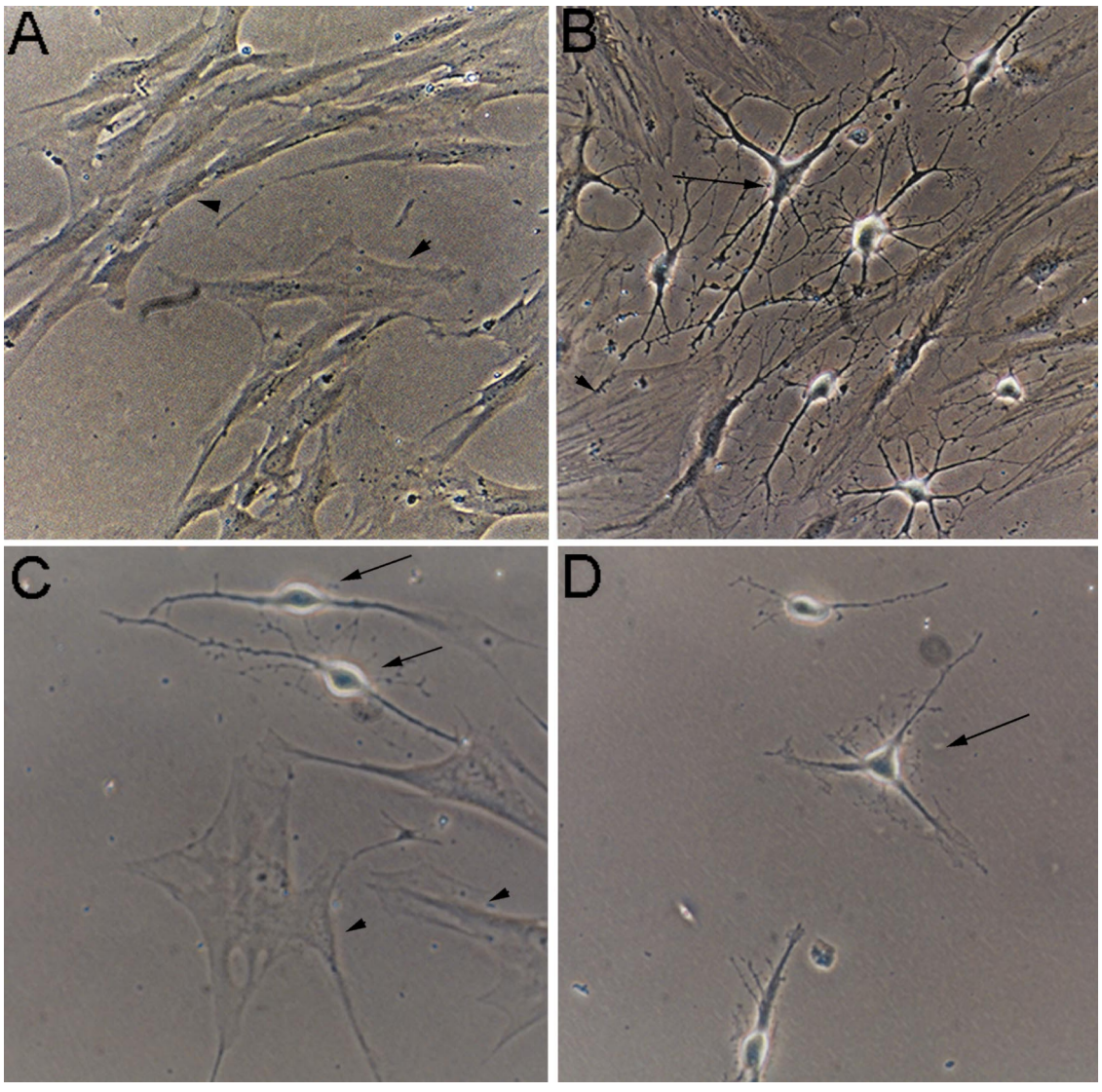

FIG. 1. Induction of neural morphology in hMSCs treated with 0.5 mM IBMX/1 mM dbcAMP. Untreated hMSCs (A); hMSCs treated with 0.5 mM IBMX/1 mM dbcAMP for 6 days (B, C, and D). Symbols: differentiated neuron-like cells (arrow); undifferentiated hMSCs (arrowhead). Photographs were taken at 200× magnification.

dium was added and replaced every 3 or 4 days. The cells were grown to 70–90% confluency over about 14 days. The cells (from passage 0) were harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 75-cm^2 flasks (Falcon) at 5000 cells/cm^2, and again grown to near confluency. The cells (from passage 1) were harvested with trypsin/EDTA, suspended at 1–2 × 10^6 cells/ml in 5% DMSO and 30% FBS, and frozen at 1 ml aliquots in liquid nitrogen. To expand a culture, a frozen stock of hMSCs was thawed, plated at 5,000 cells/cm^2, grown to 70–90% confluency over about 3–7 days. The cells (from passage 2) were harvested with trypsin/EDTA and diluted 1:3 per passage for further expansion.

Neuronal differentiation protocol. A stock solution to provide a final concentration of 0.5 mM IBMX (Sigma)/1 mM dbcAMP (Sigma) was added to 10 ml complete culture medium containing 1 × 10^6 hMSCs (passage 2) in a 58-cm^2 tissue culture dish (Falcon). IBMX/dbcAMP and complete culture medium were replaced at day 3 and the incubation continued until day 6.

Western blot analysis. Cells were rinsed with cold phosphate buffered saline (PBS) twice and drained. Whole cell lysates were prepared by adding 0.5 ml detergent based cell lysis buffer [1% (w/w) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, prepared in PBS] plus leupeptin (final concentration is 0.1 mg/ml, freshly added, Sigma), and scraping the cells into a centrifuge tube. The cells were further lysed by flushing them 3 times in a 1 ml syringe with a 21-gauge needle, and phenylmethylsulfonyl fluoride (final concentration is 574 μM, prepared in isopropanol, Sigma) was added. The sample was incubated on ice for 45 min, centrifuged at 15,000*g* for 30 min at 4°C, and the supernatant was collected. Protein content was assayed colorimetrically (Micro Protein kit, Sigma). Five μg of the cell lysate were loaded onto a 4 to 10 or 4 to 20% polyacrylamide gel. After electrophoresis, the protein was transferred by electroelution onto a nitrocellulose membrane. Immunodetection was performed with rabbit anti-NSE (ICN Biomedicals, 1:10,000 dilution), mouse anti-vimentin (DAKO, 1:1,000 dilution), rabbit anti-MAP1B (Ref. 10; 1:10,000 dilution), mouse anti-TuJ-1 (BabCo, 1:2000 dilution), mouse anti-α-tubulin (Sigma, 1:4,000 dilution), rabbit anti-neurofilament M (NF-M, Chemicon International, Inc., 1:1000 dilution), mouse anti-MAP2 (2a + 2b, Pharmingen, 1:1000 dilution), mouse anti-tau

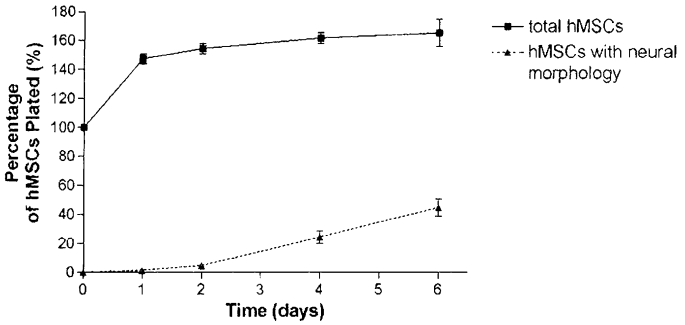

FIG. 2. Cellular proliferation and neuronal differentiation in hMSCs treated with 0.5 mM IBMX/1 mM dbcAMP. Data represent means ± SD of the experiments performed in triplicate. For quantitative analysis, we scored cells with refractile cell body, multipolar processes, and growth cone-like structures as hMSCs with neural morphology (see Figs. 1B, 1C, and 1D).

(Tau-2, Pharmingen, 1:500 dilution), mouse anti-S-100 (Neomarkers, 1:500 dilution), mouse anti-human GFAP (DAKO, 1:500 dilution), and mouse anti-myelin basic protein (MBP, Chemicon International, 1:1,000 dilution). The secondary antibody was horseradish peroxidase conjugated to either goat anti-rabbit IgG or anti-mouse IgG. The membranes were processed using enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech). About 0.5 μg human brain extract (Clontech) was used as a control.

RESULTS

Induction of Neural Morphology on hMSCs

hMSCs (Fig. 1A) were induced to differentiate in culture by incubation with 0.5 mM IBMX/1 mM dbcAMP. Typical neuron-like cells were identified as early as two days later (Figs. 1B, 1C, and 1D). After 6 days, neuron-like cells accounted for about 25% of the total population (Fig. 2). The cells had morphological features typical of neurons such as refractile cell bodies and long branching processes with growth cone-like terminal structures that frequently made contact with undifferentiated hMSCs. There was a reduced rate of cellular proliferation, but there was no obvious evidence of cell death. However, after IBMX/dbcAMP was withdrawn from the complete culture medium of the hMSCs that were treated for 6 days, all neuron-like cells died within several days. The remaining cells stopped dividing and showed senescence morphology. The data suggested that the differentiation was not reversible.

Biochemical Analysis of Cell Phenotype

Using Western blot assays, we found that the untreated hMSCs (11) expressed several markers characteristic of neural cells such as MAP1B, NSE, TuJ-1 and vimentin (Fig. 3). Using α-tubulin as a control, we found that the expression levels of both NSE and vimentin were increased after incubation with 0.5 mM IBMX and 1 mM dbcAMP. The increase in NSE and vimentin mRNAs coincided with the appearance of neuron-like cells in the cultures. However, there was no change in the expression level of either MAP1B or TuJ-1 (Fig. 3). Since NSE, MAP1B, and TuJ-1 are early neuron characteristic markers, and vimentin is an early marker for glia, the data suggested that hMSCs differentiated *in vitro* into some early progenitors of either neurons or glia. We could not detect any expression of NF-M, MAP2, tau, S-100, GFAP, and MBP (Fig. 3) in either untreated or IBMX/dbcAMP-treated hMSCs.

DISCUSSION

IBMX is a phosphodiesterase inhibitor and dbcAMP is a cAMP analogue. Both agents can elevate intracellular cAMP levels. Moore *et al.* (12) found that IBMX or dbcAMP can greatly increase the extension of processes in a medulloblastoma cell line, MCD-1. The formation of long processes induced by IBMX was associated with a decrease in cell proliferation as evidenced by a reduction in numbers of cells incorporating 5-bromo-2-deoxyuridine (BrdU). Bang *et al.* (13) found that elevation of cAMP through addition of dbcAMP and IBMX induced a neuronal morphology in human prostate carcinoma cells. The changes also included increased expression of NSE, terminal differentiation, G_1 synchronization, growth arrest, and loss of clonogenicity. Cox *et al.* (14) also found agents that can elevate intracellular cAMP such as epinephrine, isoproterenol, forskolin, IBMX, and dbcAMP can induce prostate tumor cells to assume many of the characteristics of neuroendocrine cells. The cells reverted to their original phenotype when the agents were removed. With C6 glioma cells, both Sharma *et al.* (15) and Ghosh *et al.* (16) found that dbcAMP induced neural differentiation.

In the experiments presented here, we cultured MSCs under conditions that increase intracellular cAMP, and we found that a fraction of the cells in the

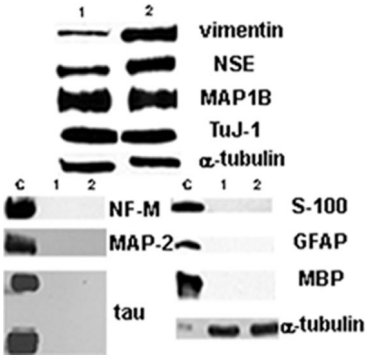

FIG. 3. Western blot assays of the expression of vimentin, NSE, MAP1B, TuJ-1, NF-M, MAP-2, tau, S-100, GFAP, MBP, and α-tubulin in hMSCs. Lane C, control, human brain extract. Lane 1, untreated hMSCs. Lane 2, hMSCs treated with 0.5 mM IBMX/1 mM dbcAMP for 6 days.

TABLE 1

Comparisons of Observations on Differentiation of MSCs[a]

Conditions:	Woodbury *et al.* (7) BME or DMSO + BHA		Sanchez-Ramos *et al.* (8) EGF/BDNF + RA or coculture		Here dbcAMP + IBMX	
Species:	Rat/human		Mouse/human		Human	
Cells with neural morphologies:	>50%		0.2 to 5%		25%	
	Cytochem. assay	Western assay	Cytochem. assay	Western assay	Cytochem. assay	Western assay
NSE	+/+++	+/+++				+/+++
NF-M	0/+++					0/0
tau	0/+++					0/0
Neu-N	0/++		+/++	++/++		
Nestin	0/+ to 0		/++	++/0		
GFAP	0/0		/++	++/++		0/0
trkA	0/+++					
Vimentin						+/+++
MAP1B						++/++
TuJ-1						++/++
MAP-2			0/0			0/0
S-100						0/0
MBP						0/0
Fibronectin			+++/+			

[a] Observations are presented as 0 to +++ scores before/after differentiation. The scores are approximations based on data presented in different formats by Woodbury *et al.* (7) and Sanchez-Ramos *et al.* (8). As indicated, parallel assays by immunocytochemistry (Cytochem.) and Western blots were not performed in many of the experiments.

cultures developed some of the phenotypic features of neural cells. The results were similar but not identical to the observations recently reported by Woodbury *et al.* (7) and Sanchez-Ramos *et al.* (8) using different culture conditions (Table 1). Similar morphological changes were seen with all three experimental conditions, but the number of neural-like cells varied widely. Our results were similar to those of Woodbury *et al.* (7) in that we saw an increased expression of NSE, but no expression of GFAP. In contrast, Sanchez-Ramos *et al.* (8) observed expression of GFAP both before and after differentiation under their conditions. We did not, however, see the expression of either NF-M or tau that Woodbury *et al.* (7) observed after differentiation. Under our conditions, there was increased expression of vimentin, as is seen in differentiation of glia. MAP1B and TuJ-1, two markers for early neurons, were expressed at about the same levels before and after differentiation. MAP-2, a marker for mature neurons, was negative. S-100 and MBP, markers for mature astrocytes and oligodendroglia, were also negative. Therefore, our results suggest that the cells differentiated into early neural progenitors under conditions that increase intracellular cAMP but not into mature cells of any specific lineage. Differentiation of the cells into mature neural cells will probably require a combination of the conditions tested to date and functional assays such as the membrane potentials of putative neurons.

In conclusion, following a treatment that elevated intracellular levels of cAMP, about 25% of hMSCs assumed a neuron-like morphology. The morphological change coincided with an increase in the expression of both NSE and vimentin. However, there was no expression of several markers for mature neurons or glia. The protocol may be useful in studying early steps of neural cell differentiation of hMSCs *in vitro* and in employing the cells for therapy of disorders of the central nervous system.

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