Journal of Cellular Biochemistry 94:645–655 (2005)

Molecular Pathways Involved in Neural In Vitro Differentiation of Marrow Stromal Stem Cells

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Abstract In recent years several reports have claimed to demonstrate trans-differentiation, namely that stem cells have been derived from a given tissue and have differentiated into phenotypes characteristic of different tissues following transplantation or in vitro treatment. For example, the mesenchymal stem cells, also referred to as marrow stromal stem cells (MSCs), present in bone marrow, have been induced to differentiate into neurons. We decided to investigate this phenomenon more in depth by a molecular and morphological follow-up. We analyzed the biochemical pathways that are currently induced to trigger neuron-like commitment and maturation of MSCs. Our studies suggest that: (i) the increase in cAMP, induced to differentiate MSCs, activates the classical PKA pathway and not through the exchange protein directly activated by cAMP (EPAC), a guanine nucleotide exchange factor for the small GTPase Rap1 and Rap2; (ii) MEK–ERK signaling could contribute to neural commitment and differentiation; (iii) CaM KII activity seems dispensable for neuron differentiation. On the contrary, its inhibition could contribute to rescuing differentiating cells from death. Our research also indicates that the currently used in vitro differentiation protocols, while they allow the early steps of neural differentiation to take place, are not able to further sustain this process. J. Cell. Biochem. 94: 645–655, 2005.

Key words: mesenchymal stem cells; cholinergic neurons; cAMP; K252a; trkA

Stem cells in the adult have traditionally been thought to be restricted in their differentiative and regenerative potential to the tissue in which they reside. However, this view has been challenged of late. Recent years have seen a number of studies that have claimed to demonstrate that trans-differentiation and somatic plasticity phenomena could be part of physiological stem cell "behavior" [Prockop, 1997; Blau et al., 2001; Slack and Tosh, 2001]. Neural stem cells, for example, have been reported to differentiate into hematopoietic cells upon peripheral injection into irradiated mice. Conversely, neural cells can be generated by marrow stromal stem cells (MSCs) both in vivo and in vitro [Armstrong et al., 2001; Blau et al., 2001; Deng et al., 2001]. Therefore, it is theoretically possible that stem cells derived from other systems, such as bone marrow, may be used for neural cell therapy. This possibility is exciting but is far from fruition, as data regarding somatic plasticity are very scant and more in-depth studies are required.

There is little doubt that bone MSCs represent one of the most accessible sources of cell therapy. The ease with which they are harvested and the simplicity of the procedure required for their culture and expansion in vitro may make them ideal candidates, although data on trans-differentiation are still limited. As such, studies aiming to well understand the biology of MSCs are highly desirable [Prockop,

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Received 25 June 2004; Accepted 3 August 2004
DOI 10.1002/jcb.20315

1997; Pittenger et al., 1999; Bianco and Gehron Robey, 2000; Woodbury et al., 2002; Seshi et al., 2003].

As far as the origin of marrow stromal cells is concerned, it should be considered that in addition to hematopoietic stem cells, bone marrow contains cells that meet criteria for stem cells of non-hematopoietic tissues. These stem cells are currently referred to either as mesenchymal stem cells because of their ability to differentiate into mesenchymal cells (such as bone and cartilage cells, adipocytes) or as marrow stromal cells, because they appear to arise from the complex array of supporting structures found in marrow [Bianco and Riminucci, 1998; Pittenger et al., 1999; Bianco et al., 2001].

MSC somatic plasticity can be easily analyzed in vitro. In fact, several reports have described conditions under which MSCs can be differentiated in culture to neuron-like cells. Several compounds have been used to induce the neural differentiation of MSCs. Some of these agents include retinoic acid combined with growth factors; beta-mercaptoetanol; noggin factor; 5-Aza-C and cytokines; factors that increase cAMP levels either in the presence or absence of K252a. The comparison of these differentiation protocols demonstrates that the best procedure by which MSCs show a neuron-like phenotype relies upon conditions that increase intracellular cAMP, such as forskolin treatment. The differentiation is further sustained by K252a treatment [Woodbury et al., 2000; Black and Woodbury, 2001; Deng et al., 2001; Sanchez-Ramos, 2002; Woodbury et al., 2002; Munoz-Elias et al., 2003; Rismanchi et al., 2003; Seshi et al., 2003; Jori et al., 2004].

K252a is an alkaloid that is structurally similar to staurosporine and isolated from *Nocardiopsis* sp. soil fungi. This compound is a general cell-permeable protein kinase inhibitor. It was demonstrated that K252a inhibits CaM kinase II, protein kinase A (PKA), and protein kinase C (PKC) [Hashimoto et al., 1991; Twomey et al., 1991; Tapley et al., 1992]. It can also act as an inhibitor of the tyrosine kinase activity of the NGF receptor trkA [Ohmichi et al., 1992; Rovelli et al., 1993]. Recently, Roux et al. demonstrated that K252a can have neuroprotective properties and can also induce the activation of the MEK–ERK pathway [Roux et al., 2002].

It is evident that K252a and forskolin can profoundly modify cell biology by acting on several biochemical pathways. We decided to investigate which of these pathways are involved in MSC neural differentiation induced by in vitro treatment with forskolin and K252a.

MATERIALS AND METHODS

Animals and MSC Cultures

In accordance with protocols by Prockop and co-workers [Colter et al., 2000; Colter et al., 2001], MSCs were harvested from the bone marrow of the femurs and tibias of 4 to 12-month-old rats by inserting a 21-gauge needle into the shaft of the bone and flushing it with complete-modified Eagle's medium (MEM) containing 20% fetal bovine serum (FBS), 2 ng/ml basic FGF (Preprotech, Rocky Hill, NJ), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (we indicated this medium as PRO for proliferating). Cells from one rat were plated onto two 100-mm dishes. After 24 h, non-adherent cells were discarded, and adherent cells representing the MSCs were washed twice with PBS. The cells were then incubated for 5–7 days to reach confluence. Cells were extensively propagated for further experiments.

All cell culture reagents were obtained from Invitrogen Italia (Milan, Italy), unless otherwise stated.

Uncommitted Cells and Neural Induction

MSCs were grown as uncommitted pluripotent cells in PRO medium (see above). Neuronal induction was performed as described by Woodbury et al. [2002] with modifications. Briefly, prior to neuronal induction, PRO medium was discarded and cells were grown overnight in pre-induction medium (PRE) composed of DMEM, 20% FBS, and 10 ng/ml bFGF. The cells were then rinsed with PBS and transferred to neuronal induction medium (NIM) consisting of 100 μM BHA, 100 μM forskolin, 2% DMSO, 25 mM KCl, 2 mM valproic acid, 1X B27 supplement (Invitrogen Italia), in a base of DMEM. Cells were maintained in NIM for up to 5 days.

We also performed differentiation in alternative media as reported in the Results section.

Cell Death Assay

We used CellTiter 96 proliferation assay and CytoTox 96 cell death assay (both from Promega, Madison, WI) according to the manufacturer's instructions. Briefly, at the beginning of

the experiment, 3,000 cells were plated in a 96-multiwell plate in PRO medium. After 24 h, cells were switched to PRE and then, after another 24 h, cells were incubated for 1 and 3 days in neural induction medium (NIM) or modifications of it. Cellular death levels were evaluated by spectrophotometrically determining the dehydrogenase activity released in the culture medium by dead cells. This was normalized with respect to cell number. Each assay was repeated at least three times.

Cell Cycle Analysis

For each assay 5×10^5 cells were collected and re-suspended in 500 μl of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 μg/ml propidium iodide, RNAse A). Cells were incubated in the dark for 30 min and then analyzed. Samples were acquired on a FACS-Calibur flow cytometer using the Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) and analyzed with standard procedure using the Cell Quest software and the Mod-FitLT software version 3 (Becton Dickinson).

Immunocytochemistry

We determined the percentage of neurons in MSC cultures by growing cells on glass coverslips. At different times after the chosen treatments, coverslips were fixed with 4% paraformaldehyde for 15 min followed by three washes in PBS. Slides were then treated with 0.3% H_2O_2 in methanol for 5 min, followed by washes with 1% BSA in PBS. At this point, the cells were incubated with primary antibody (clone NN18 from Sigma-Aldrich Italia, Milan, Italy) targeted against pan-neurofilaments (diluted 1/100 in PBS supplemented with 1% BSA) for 90 min at room temperature (RT). Afterwards, slides were washed three times with PBS and incubated with goat anti-mouse secondary antibodies conjugated to peroxidase (DAKO, Carpinteria, CA) for 45 min at RT. Finally, after further washes in PBS, slides were treated with DAB substrate (Roche, Milan, Italy).

Acetylcholinesterase (AChe) Cytochemistry

AChe in situ detection was performed according to the protocol of Zhang et al. [2002]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min, after which they were rinsed with PBS. The cultures were then incubated in a fresh solution consisting of 3 mM copper sulfate, 5 mM sodium citrate, 0.5 mM potassium ferricyanide, and 1.8 mM acetylthiocholine in 0.1 M phosphate buffer pH 6, for 1–2 h at 37°C. After two rinses with 0.5 mM Tris-HCl pH 7.6, the cultures were incubated for 5–10 min in an intensification solution composed of DAB substrate (Roche, Germany).

TUNEL Assays and Determination of Apoptotic Index

The cells for TUNEL assays (in situ cell death detection kit from Roche, Germany) were grown on glass coverslips. Cells were fixed for 15 min using 4% paraformaldehyde and the TUNEL reaction was performed according to the manufacturer's instructions. The apoptotic index was calculated by the number of positive TUNEL cells out of 1,000 cells in five different microscopic fields.

RNA Extraction and RT-PCR

Total RNA was extracted from cell cultures using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The mRNA levels of the genes under analysis were measured by RT-PCR amplification, as previously reported [Galderisi et al., 1999].

Sequences for human mRNAs from the GeneBank (DNASTAR, Inc., Madison, WI) were used to design primer pairs for RT-PCR reactions (OLIGO 4.05 software, National Biosciences, Inc., MN). Appropriate regions of HPRT cDNA were used as controls. PCR cycles were adjusted to have linear amplification for all the targets. Each RT-PCR reaction was repeated at least three times. A semi-quantitative analysis of mRNA levels was carried out by the "GEL DOC UV SYSTEM" (Biorad Company, CA).

Statistical Analysis

Statistical significance was evaluated using ANOVA analysis followed by Student's *t*- and Bonferroni's tests.

RESULTS

We performed an initial study investigating the pathways that could be involved in neural differentiation induced by NIM treatment. To this end, we induced differentiation in the presence of specific pathway inhibitors. Three days after the induction of differentiation, we carried out morphological, immunocytochemistry, and

TABLE I. In Vitro Cell Differentiation, Cell Death Assays, and Cell Cycle Analysis of MSCs Incubated Under Different Experimental Conditions

	Panel A[a]			Panel B[b]		
	Inhibitor or analog	Cell differentiation	Cell death	G_0/G_1 (%)	S (%)	G_2/M (%)
NIM		+++	+	81.55	10.54	7.91
NIM minus K252a		++/+++	+	81.26	10.61	8.13
NIM cAMP analog	8-pCPT-cAMP 100 μM	no differentiation	+	83.18	9.30	7.52
NIM PKA⊥	KT5720 0.5 μM	no differentiation	+++++			
NIM PKC⊥	Chelerytrine 20 μM	no differentiation	+++++			
NIM trkA⊥	AG879 25 μM	no differentiation	+++++			
NIM CaMKII⊥	KN-62 0.1 μM	++	+	81.81	8.80	9.39
NIM PI3K⊥	LY294002 20 μM	++/+++	+/++	81.30	14.46	4.24
NIM MEK⊥	PD98,059 30 μM	+	+	81.14	9.15	9.71

[a]The neural induction medium with K252a (NIM) was chosen as a reference. Other investigations were performed with NIM lacking K252a (NIM minus K252a). In one experiment, the forskolin was replaced with 8-pCPT-2′O-Me-cAMP, which is an analog of natural cAMP (NIM cAMP analog). Under other assay conditions, the NIM did not contain K252a and different kinase inhibitors were added to the medium: PKA inhibitor (NIM PKA⊥), PKC inhibitor (NIM PKC⊥), CaM KII inhibitor (NIM CaM KII⊥). Finally, in one experiment, the classical NIM containing K252a was supplemented with PI3K inhibitor (NIM PI3K⊥) or with MEK inhibitor (NIM MEK⊥). Cell differentiation assays: At 3 days of incubation in differentiating media the percentage of neuron-like cells was determined by anti-neurofilament immunostaining. Cell death assay: The table presents data obtained after 3 days of incubation in differentiating media. The percentages of cell differentiation and cell death were indicated as follows (+) <20%; (++) 20%–40%; (+++) 40%–60%; (++++) 60%–80%; (+++++) >80%.

[b]Flow cytometry analysis of MSCs after 3 days of incubation in differentiating media. For each experimental condition the percentage of live cells in the different phases of cell cycle is shown.

flow cytometry analyses to compare the degree of differentiation observed under the different experimental conditions with that observed in classical NIM medium, chosen as a reference (Table I). In addition, we performed a cytotoxicity test to evaluate the survival rate in the different media.

At day 1 of incubation with NIM the differentiation process had overlapping features both in the presence and absence of K252a (data not shown). However, at day 3 we observed 10%–15% of cell reversion in the absence of K252a compared to cultures incubated with complete NIM. Moreover, K252a increased the survival rate (Table I).

Canonical PKA Pathway Is Required for Neural Differentiation of MSCs

PKA is a general receptor for cAMP that causes the phosphorylation of several targets. Recently, Rangarajan et al. identified PKA-independent pathways [Rangarajan et al., 2003], which act through the exchange protein directly activated by cAMP (EPAC), a guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2 [de Rooij et al., 2000]. We induced neural differentiation of MSCs with NIM medium in which forskolin was replaced with 100 μM 8-pCPT-2′O-Me-cAMP. This compound is an analog of natural cAMP, which specifically activates EPAC-dependent pathways and not those related to PKA. Treatment with this cAMP analog did not induce any neural differentiation; however, the survival rate and the cell cycle profile were similar to that observed in cells treated with unmodified NIM (Table I).

We then treated cells with NIM lacking K252a and in the presence of 0.5 μM KT5720, a specific inhibitor of PKA [Sung et al., 2001]. At 2 days of treatment, almost 90% of the cells were already dead and no sign of differentiation was observed (Table I). The treatment of undifferentiated MSCs with KT5720 did not induce cell death (data not shown), suggesting that only in specific phases of cell life can PKA affect cell survival.

PKC Is Required for Cell Survival

We inhibited PKC by treating cells in NIM without K252a and supplemented with 20 μM chelerytrine [Herbert et al., 1990; Colombo and Bosisio, 1996]. After 1 day of treatment, more than 90% of the cells were already dead and no differentiation was observed (Table I). As a control, we treated proliferating MSCs with chelerytrine. Also in this case we observed a significant modification of cell survival rate. These data suggest that PKC has a key role in regulating MSC cell survival under different experimental conditions.

Differentiated MSCs Have the trkA Receptor and Are Sensitive to its Inhibition

Several authors [Ohmichi et al., 1993; Rovelli et al., 1993] reported that K252a can specifically

block the trkA^{p140} NGF receptor. To verify this, we treated cells with NIM without K252a and in the presence of 25 μM AG879 to inhibit the trkA receptor [Ohmichi et al., 1993]. Under these conditions we observed no sign of cell differentiation and an extremely high rate of cell death (up to 80%) (Table I). The AG879 treatment did not affect the survival of MSCs grown in proliferating medium (data not shown).

These data suggest that the differentiation effect of K252a does not rely on its ability to inhibit the trkA receptor. Moreover, these results indicate that signaling through the trkA receptor could have a role in MSC maturation toward a neuron-like phenotype.

These different outcomes of AG879 treatments prompted us to perform a follow-up of trkA expression during in vitro differentiation of MSCs. We monitored trkA mRNA expression profiles for 5 days following neural induction according to the protocol reported in the Materials and Methods section (Fig. 1). In undifferentiated MSCs (PRO medium) and in those incubated under pre-induction conditions (PRE medium) we did not detect trkA mRNA. This result is in agreement with the absence of biological effects of trkA inhibition on undifferentiated MSCs. On the contrary, in differentiating cells, the trkA gene showed a high expression at 1 day of differentiation (1 NIM medium) and then decreased (Fig. 1).

Since the activity of trkA is triggered by NGF, we also looked for the expression of NGF mRNA. The NGF mRNA level was high in undifferentiated cells (PRO medium) and in those incubated in PRE, then it decreased significantly at 1 day of differentiation and rose again at 2 and 5 days in NIM (Fig. 1).

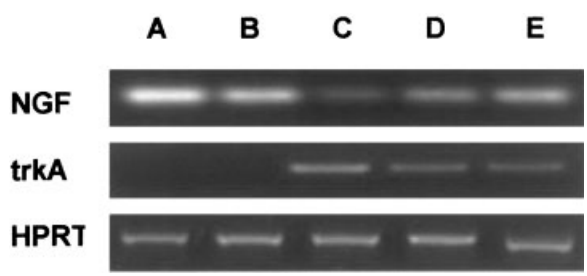


Fig. 1. mRNA expression levels in MSCs cultured for different times and in different media. The mRNA levels were measured by a GELDOC instrument and normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. **Top panel**: Semiquantitative RT-PCRs performed to detect trkA and NGF mRNAs. Cells were grown in proliferating medium (**A**). Then they were incubated for 24 h in pre-induction medium (**B**). Finally, cells were grown for 1 (**C**), 2 (**D**), and 5 (**E**) days in NIM (neural induction medium).

Ca MK II Inhibition Did Not Significantly Affect the Neural Differentiation of MSCs

The contribution of K252a to neural differentiation of MSCs should not be attributed to the block of PKA, PKC, and trkA activity; since we observed that the inhibition of these molecules profoundly affected cell survival of differentiating MSCs. On the other hand, the 3 nM concentration of K252a in NIM could allow only partial PKA and PKC inhibition. In fact, under some experimental conditions, the inhibition constant (Ki) of K252a for PKA and PKC was 17 nM and 18 nM, respectively [Kase et al., 1987]. Moreover, some authors reported that, in some cell types, 3 nM K252a inhibited 50% (IC_{50}) of the trkA activity [Tapley et al., 1992].

We induced differentiation of MSCs in NIM without K252a and in the presence of 0.1 μM KN-62, which specifically inhibits CaM KII activity [Sung et al., 2001]. MSCs induced to differentiate in NIM without K252a and in the presence of KN-62 showed a cell survival rate and a percentage of differentiated cells comparable to those observed when differentiation was induced in classical NIM (containing K252a) (Table I). In addition, the growth arrest in G_0/G_1 of differentiating MSCs was not affected by inhibition of CaM KII enzyme (Table IB).

Phospatidylinositol 3-Kinase (PI3K) Blockade Affects Cell Survival and MEK–ERK Inhibition Impairs Cell Differentiation

Roux et al. demonstrated that k252a has neuroprotective activity through the induction of PI3K. We evaluated this effect by treating MSCs with NIM devoid of k252a and in the presence of a specific PI3K inhibitor (20 μM LY294002) [Sung et al., 2001]. We observed a significant (−25%) reduction of cell survival at the third day of differentiation (Table I). No modification of cell growth arrest was observed (Table IB).

K252a can also induce the MEK–ERK pathway [Roux et al., 2002]. For this reason, we induced MSC differentiation in complete NIM (containing k252a) with an inhibitor of MEK–ERK cell signaling (30 μM PD98,059) [Sung et al., 2001]. At 1 day of treatment the percentage of differentiated neurons was similar both

in cells incubated with classical NIM and in those grown in NIM plus PD98,059. Nevertheless, at 3 days, almost 40% of differentiated neurons reverted to uncommitted cells in cultures containing the MEK–ERK inhibitor (Table I). It is noteworthy that the reversion of differentiation was not associated with a reentry into cell cycle (Table IB).

In-Depth Analysis of CaM KII and MEK–ERK Signaling

We decided to better evaluate if CaM KII inhibition could have the same effects as k252a on MSC differentiation. Moreover, we also wanted to assess the role of the MEK–ERK pathway in neural maturation of MSCs.

We performed a cytochemical and molecular analysis of rat MSCs to monitor neural differentiation and cell death for 3 days in vitro following incubation with specific inhibitors of these pathways. We performed morphological analyses of differentiating MSCs and identified differentiating neurons by anti-neurofilament immunostaining. Moreover, having demonstrated that MSCs differentiating into neurons acquire a cholinergic phenotype [Jori et al., 2004], we performed cytochemical AChe stainings to identify cholinergic neurons.

We performed neurofilament (NF) immunostaining and AChe cytochemical detection at 1 and 3 days in NIM, since we had already demonstrated that this is a critical time period for neural in vitro differentiation of MSCs [Jori et al., 2004].

At 1 day in NIM more than 90% of the cells were positive for both NF and AChe staining in all of the different media assayed. At 3 days in NIM, we observed a significant reduction in the number of cells showing a neuron-like phenotype, indicating that all experimental conditions tested allow for an initial neuronal differentiation, but are not able to further sustain this process (Fig. 2). However, while cells differentiated in complete NIM and in NIM with CaM KII inhibitor and without K252a showed similar levels of cells positive for NF and AChe staining, cells differentiated in the presence of MEK–ERK inhibitor had a reduced number of neuron-like cells (Fig. 2).

We used RT-PCR to analyze the expression of the differentiation markers. Neuron specific enolase (NSE) and Id2 proved to be the more suitable for monitoring in vitro neural commitment and differentiation, since they showed a

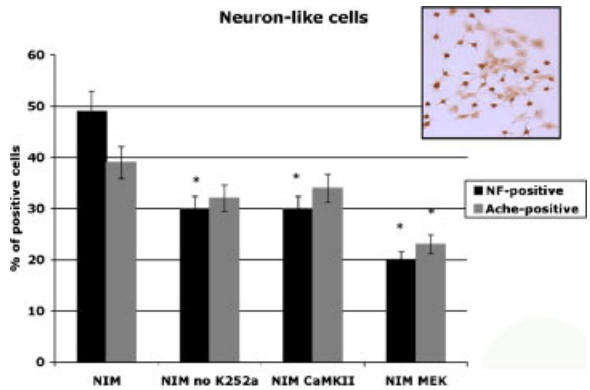


Fig. 2. In situ AChe activity detection. The assays were carried out on MSCs that were incubated in several differentiating media. The neural induction medium with K252a (NIM) was chosen as a reference. Other investigations were performed with NIM lacking K252a (NIM minus K252a). In another assay the NIM did not contain K252a and was supplemented with CaM KII inhibitor (NIM CaM KII⊥). Other experiments were carried out in NIM containing K252a and in the presence of MEK inhibitor (NIM MEK⊥). The percentage of AChe-positive cells was determined by counting at least 1,500 cells in five to six different microscopic fields. A typical AChe-positive cell is shown in the inset. $^*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

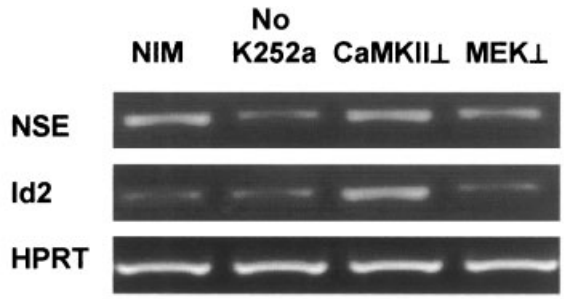
progressive increase (NSE) or decrease (Id2) in mRNA levels during the experimental differentiation process [Jori et al., 2004].

NSE gene expression was lower in MSCs that were differentiated in the presence of MEK inhibitor compared to cells incubated in classical NIM. On the other hand, the NSE mRNA levels did not change significantly when differentiation was performed with CaM KII inhibitor (Fig. 3).

Id2 mRNA was higher in cells treated with CaM KII inhibitor than in those incubated in classical NIM (Fig. 3), suggesting that, at least in part, the blockage of CaM KII activity could impair the neural maturation. The inhibition of MEK–ERK signaling did not affect the expression of Id2 mRNA compared to controls (Fig. 3).

Molecular and Morphological Analysis of Cell Death Under the Different Experimental Conditions

In a preliminary screening, performed using a cytotoxic assay, we evaluated the rate of cell death under different experimental conditions (Table I). The results of these assays showed that, besides the high cell death rate observed in the presence of either PKA, PKC, or trkA inhibitors, the blockage of CaM KII- and MEK-dependent pathways can also affect



	NIM	No K252a	CaMK2⊥	MEK⊥
NSE	4.50 ± 0.60	2.45 ± 0.25	5.13 ± 0.70	3.13 ± 0.40
Id2	1.52 ± 0.20	1.38 ± 0.28	2.71 ± 0.30	1.11 ± 0.15

Fig. 3. mRNA expression levels in MSCs cultured under the same experimental conditions described in Figure 2. The mRNA levels were measured by a GELDOC instrument and normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. **Top panel**: Semiquantitative RT-PCRs performed to detect neuron specific enolase (NSE) and Id2 mRNAs. **Lower panel**: Densitometric values of semiquantitative RT-PCR analyses. The variations in gene expression are given as arbitrary units.

cell survival. To gain further insight into this phenomenon, we evaluated the apoptotic rate in differentiating MSCs incubated either with KN-62 or PD098,059, respectively. The apoptotic index was significantly lower ($P < 0.05$) in cells treated with KN-62, compared to controls in NIM (12% vs. 20%) (Fig. 4), suggesting that the impairment of CaM KII could have an impact on programmed cell death. We did not observe differences between cells differentiated in the presence of PD098,059 with respect to NIM (Fig. 4).

We also performed a molecular study to analyze the expression of genes involved in apoptosis. To this end, we detected mRNA variation of death-inducing and death-inhibitory genes such as, BCLX-$_L$ and BCL-2 with anti-apoptotic activity, and BAX or BCLX-$_S$, which have pro-apoptic features and play a central role in regulating apoptosis. The ratio of death agonists to death antagonist genes can determine cell fate (Reed, 1997; Adams and Cory, 1998). In cells differentiated in the presence of CaM KII inhibitor, the ratio of pro-/anti-apoptotic genes (Bax/Bcl-2 and $BclX_S/BclX_L$) is lower compared to cells grown in NIM (Fig. 5). In cells

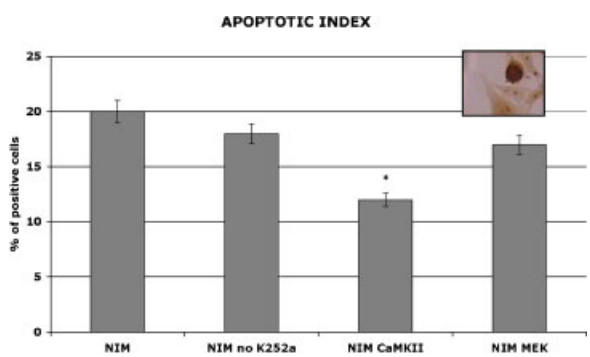


Fig. 4. Apoptotic index determined by TUNEL assays carried out on MSCs cultured under the same experimental conditions described in Figure 2. The percentage of TUNEL-positive cells was determined by counting at least 1,000 cells in five to six different microscopic fields. A typical TUNEL-positive cell is shown in the inset. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

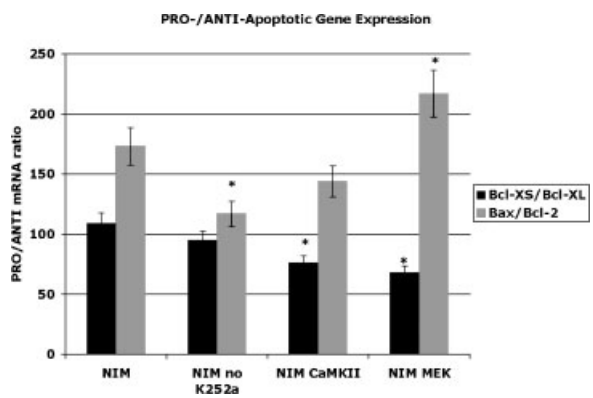


Fig. 5. Semi-quantitative RT-PCR analysis of the Bcl-X_L/Bcl-X_S ratio and Bcl2/Bax ratio in MSCs cultured under the same experimental conditions described in Figure 2. The mRNA levels were measured by a GELDOC instrument and normalized with respect to HPRT, chosen as an internal control. Subsequently, the relative expression units were used to obtain the anti-/pro-apoptotic ratios that are shown in the figure. Each experiment was repeated at least three times.

differentiated in NIM without K252a, we also observed a decrease in pro-/anti-apoptotic ratios compared to cells incubated in complete NIM. On the contrary, in cells differentiated in the presence of MEK inhibitor, the decrease of the $BclX_S/BclX_L$ ratio was compensated by an Bax/Bcl-2 increase (Fig. 5).

DISCUSSION

The development of "cell therapy" for nervous system diseases by NSCs is hampered by the technical difficulties in harvesting autologous NSCs. However, neural cells can be generated by MSCs either in vivo or in vitro. Therefore, it is theoretically possible that stem cells derived from other systems, such as bone marrow, may be used for neural cell therapy. There is little doubt that bone MSCs represent one of the most accessible sources for cell therapy. The ease with which they are harvested (a simple marrow aspirate) and the simplicity of the procedure required for their culture and expansion in vitro may make them ideal candidates. This possibility is exciting, but is far from fruition, as data on trans-differentiation are very scant and more in-depth studies are required.

We decided to study the in vitro differentiation protocol that appears to be, at the moment, the best procedure by which MSCs show a neuron-like phenotype.

Neural cell differentiation of MSCs is primarily induced by an increase in intracellular cAMP through forskolin treatment. This maturation process is further sustained by K252a treatment that, however, cannot induce cell differentiation per se.

We performed a more in-depth investigation of the pathways that are involved in K252a and forskolin treatment. First, we aimed to determine if the increase in cAMP triggers the classical PKA-dependent signal transduction or the alternative EPAC–RAP pathway, which has been suggested to be involved in neural cell differentiation [Bos, 1998; de Rooij et al., 2000]. Therefore, in the classical NIM, we replaced the forskolin with 8-pCPT-2′O-Me-cAMP. This molecule specifically activates EPAC-dependent pathways and not those dependent on PKA [Rangarajan et al., 2003]. We did not observe any cell differentiation under these experimental conditions, suggesting that the cAMP molecules induced by forskolin are recruited in the classical PKA-dependent pathway. On the other hand, cell treatment with NIM (containing forskolin) in the presence of PKA inhibitors did not induce cell differentiation. Furthermore, the inhibition of this biochemical pathway induced massive cell death, suggesting that PKA has a role both in cell survival and in cell differentiation.

Next, we investigated the molecular pathways that can be affected by K252a treatment. K252a can inihibit PKA, PKC, Ca MK II, and trkA receptor [Hashimoto et al., 1991; Twomey et al., 1991; Ohmichi et al., 1992; Tapley et al., 1992; Rovelli et al., 1993]. On the contrary, K252a activates ERK through MEK and AKT through PI3K [Roux et al., 2002]. Treatment of differentiating MSCs with PKA-, PKC-, and trkA-specific inhibitors induced massive cell death, while it had negligible effects on cell survival of proliferating MSCs. Our data strongly suggest that during neural differentiation of MSCs these signaling pathways have a main role in regulating cell survival. Moreover, it can be excluded that the pro-differentiating effects of K252a are due to the blockage of these pathways.

On the contrary, K252a can contribute to rescuing differentiating cells from death by activating the AKT-PI3K pathway. In fact, cells differentiating in NIM with K252a and in the presence of the PI3K inhibitor LY294002 showed an increase in cell death compared to cells differentiating in classical NIM.

It is interesting that trkA inhibition induced MSC death, suggesting that these cells have typical neuron features. The increase in trkA expression level in differentiating MSCs along with the decrease in NGF mRNA seems to indicate that MSCs could contribute, through an autocrine paracrine mechanism, to neural differentiation that has been induced by an increase in cAMP levels. Furthermore, as differentiation proceeds, MSCs appear to switch from "producers" to "consumers" of NGF.

MSCs incubated in NIM without K252a and in the presence of CaM KII inhibitor, showed a degree of neural differentiation and a survival rate similar to that obtained with NIM containing K252a (Figs. 3 and 4). These data suggest that the pro-neural effects of K252a could be ascribed, at least in part, to its ability to block the CaM KII enzyme. The results are somewhat surprising, since several reports demostrated that CaM KII activity was induced during in vivo and in vitro neural differentiation [Jensen

et al., 1991; Donai et al., 2000; Bui et al., 2003]. However, Thashima et al. demonstrated that overexpression of CaM KII inhibits neurite outgrowth of PC12 cells [Tashima et al., 1996]. This suggests that the calmodulin kinases could have multiple scheduled expression profiles in the different neural cells and/or in some phases of neural differentiation. The change in CaM KII can serve to regulate the neuron sensitivity to calcium ions. Neuronal survival in vitro is promoted by KCl in the medium, which stimulates bioelectric activity and calcium uptake through voltage-sensitive Ca^{2+} channels. A sustained KCl depolarization of the cell membrane could induce a high increase in the intracellular calcium level that in turn could induce cell death; the modulation of CaM KII activity should prevent prolonged calcium waves from inducing programmed cell death. In accordance with this hypothesis, we detected a lower cell death rate in cells grown in NIM devoid of K252a and supplemented with CaM KII inhibitor compared to complete NIM (Fig. 4). Also, molecular data on the expression levels of pro- versus anti-apoptotic genes are in agreement with this hypothesis (Fig. 5). It must be pointed out that also in cells induced to differentiate in NIM without K252a, we observed a decrease in pro-/anti-apoptotic ratios compared to cells incubated in complete NIM (Fig. 5). However, we observed a comparable number of TUNEL-positive cells under these two different conditions (Fig. 4). These data suggest that K252a could have an anti-apoptotic impact due to its blocking effect on CaM KII activity, but this phenomenon could be in part counteracted by the other K252a activities.

The inhibition of the MEK–ERK pathway has a great effect on the neural differentiation potential of MSCs. In fact, MSCs having neuron-like morphology decreased significantly in the presence of MEK–ERK inhibitors (Fig. 3). The molecular data are in agreement with morphological observations (Fig. 4).

The importance of this pathway could be further underlined by the observation that, besides K252a, several other components of NIM can contribute to the activation of MEK–ERK signaling [Yuan et al., 2001; Hansen et al., 2003].

In a previous study, we observed that 28% of differentiating cells were positive after 1 day in NIM [Jori et al., 2004]. These results overlapped with those regarding differentiating cholinergic neurons, which were identified by cytochemical AChe staining. These percentages were observed also at 3 days in NIM. In this study, however, more than 90% of the cells were positive for NF and AChe at 1 day in NIM, and at 3 days in NIM we observed a slightly higher percentage of neuron-like cells compared to previous studies (39% NF-positive cells vs. 28%).

The differences could be ascribed to the presence of basic FGF in the proliferating medium. We added this cytokine to the growth medium to obtain an effective in vitro expansion of MSCs as already reported [Bianchi et al., 2003]. However, basic FGF, besides its growth promoting activities, could contribute to the neural commitment of undifferentiated stem cells [Palmer et al., 1999]. Thus, basic FGF could contribute to the switch of MSCs toward neuron-committed cells.

CONCLUSIONS

The molecular and cellular follow-ups of in vitro neural differentiation of MSCs under different experimental conditions suggest that:

1. The increase in cAMP induced by forskolin treatment activates the classical PKA pathway;
2. MEK–ERK signaling could contribute to neural commitment and differentiation;
3. CaM KII activity seems dispensable for neuron differentiation. On the contrary, its inhibition could contribute to rescuing differentiating cells from death.

Our study suggests that these in vitro differentiation protocols, while they allow the early steps of neural differentiation to take place, are not able to further sustain this process and hence are not useful for long-term in vitro survival of neurons. Further studies are highly desirable to design experimental protocols more amenable to obtain adequate in vitro neural differentiation of MSCs.

ACKNOWLEDGMENTS

We thank M. Basso of the Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, PA, for editing the manuscript.

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