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Suppressing Phosphatidylcholine-Specific Phospholipase C and Elevating ROS Level, NADPH Oxidase Activity and Rb Level Induced Neuronal Differentiation in Mesenchymal Stem Cells

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Abstract In the previous research, we found that D609 (tricyclodecan-9-yl-xanthogenate) could induce human marrow stromal cell (hMSC) differentiation to neuron-like cells. In this study, to understand the possible mechanism, we sequentially investigated the changes of phosphatidylcholine-specific phospholipase C (PC-PLC) activity, the expression of Rb, the intracellular reactive oxygen species (ROS) levels, NADPH oxidase and superoxide dismutase (SOD) activities when D609 induced neuronal differentiation in rat mesenchymal stem cells (MSCs). The results showed that D609 obviously inhibited the activity of PC-PLC when it induced neuronal differentiation in rat MSCs. Simultaneously, ROS level and the activity of NADPH oxidase increased significantly, but the MnSOD and Cu/ZnSOD activities were not altered. Furthermore, the level of Rb protein was evidently elevated. Our data suggested that PC-PLC mediated neuronal differentiation of rat MSCs by elevating NADPH oxidase activity, ROS level, and up-regulating the expression of Rb protein. J. Cell. Biochem. 100: 1548–1557, 2007.

Key words: mesenchymal stem cells; PC-PLC; neuronal differentiation; ROS

It has been demonstrated that mesenchymal stem cells (MSCs) are capable of differentiating into neurons and astrocytes in vitro and in vivo [Prockop, 1997; Pittenger et al., 1999]. It was reported that the increase in intracellular cAMP, which activated the classical PKA pathway and MEK–ERK signaling, induced the neural differentiation of MSCs [Jori et al., 2005b]. However, whether there are other signaling pathways or key elements that are involved in the neural differentiation of MSCs are not known. In our previous research, it was found that D609 (tricyclodecan-9-yl-xanthogenate) could induce human vascular endothelial cell (hVEC) and human marrow stromal cell (hMSC) differentiation into neuron-like cells [Wang et al., 2004]. However, the possible mechanism by which D609 induced MSC neural differentiation is not known. Our purpose of this study is to answer this question so as to more clearly understand the mechanism of the neural differentiation in MSCs.

D609 has been known as a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC) and the signal transduction via PC-PLC is important in cell proliferation and differentiation in hVECs and some other kinds of cells [Amtmann, 1996; Li et al., 1998; Andrei et al., 2004], we proposed that PC-PLC might play a key role during MSC differentiation into neurons. To confirm this hypothesis, we first examined the changes of PC-PLC activity in the neural differentiation of rat MSCs.

Except as a specific inhibitor of PC-PLC, D609 also is an antioxidant or reducer, and could

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inhibit the accumulation of intracellular reactive oxygen species (ROS) [Zhou et al., 2001; Lauderback et al., 2003]. It has been reported that ROS act as intracellular messengers in cell differentiation signaling pathways [Shibata et al., 2003]. Given recent studies on ROS, under physiological conditions, the level of ROS in newly born cortical neurons was much higher than that in neural progenitor or glial cells, and under appropriate culture conditions, the level of intracellular ROS was higher in neuronal cells than that in proliferative progenitor cells [Tsatmalia et al., 2005]. The finding that neurons have higher level of ROS than their progenitors is consistent with the possibility that ROS play a regulatory role in neuronal differentiation. To understand whether ROS are implicated in the neural differentiation of rat MSCs, we investigated the changes of the intracellular ROS level after PC-PLC was suppressed by D609.

There are at least two pivotal enzymes associated with the regulation of ROS production, NADPH oxidase and antioxidant enzyme superoxide dismutase (SOD). ROS generated by the activated NADPH oxidase could act mainly as intracellular messengers in signal transduction pathways [Sauer et al., 2001]. Conversely, the enzyme SOD is a primary cellular defense against ROS [Ebert et al., 2006]. To know whether the level of ROS is regulated by NADPH oxidase and SOD in the neural differentiation of rat MSCs, we checked the activity changes of these two enzymes after PC-PLC was inhibited by D609.

It has been demonstrated that retinoblastoma (Rb) plays a significant role in neural cell differentiation. The level of Rb is up-regulated when embryonal carcinoma cells were induced to differentiate into neural tissues in vitro [Slack et al., 1993]. Rb protein is known as a key factor in the neural differentiation of several systems [Garriga et al., 1998; Lipinski and Jacks, 1999]. To understand whether Rb protein participates in the neural differentiation of rat MSCs, we investigated the changes of Rb expression after PC-PLC was suppressed by D609.

Here, we report that suppressing the activity of PC-PLC by D609 induced rat MSC neuronal differentiation. During the MSC differentiation, intracellular ROS level and the activity of NADPH oxidase in differentiated MSCs were significantly higher than that in undifferentiated MSCs, but the activities of SOD were not altered. Moreover, the expression of Rb protein was obviously up-regulated.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL Co., Grand Island, NY. D609, L-α-phosphatidylcholine (from egg yolk) and DCHF (2′, 7′-dichlorofluorescin) were purchased from Sigma, Co. Fetal bovine serum (FBS) was obtained from Hycolon Lab, Inc. Fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated antibodies CD14, CD29, CD34, CD44, CD45, CD105, HLA-ABC, HLA-DR were purchased from BD Pharmingen, San Diego, CA. Primary antibodies (Rabbit anti-rat neuron-specific enolase (NSE), neurofilament-L (NF), synapsin and secondary antibody (FITC-goat anti-rabbit IgG)) were purchased from Santa Cruz Co. Rabbit anti-rat Rb and HRP-anti rabbit IgG were purchased from Zhongshan Co. SOD detection kit was purchased from Nanjing Jiancheng Co. All other reagents were ultrapure grade.

Cell Culture

Rat MSCs were isolated from the femurs and tibias of male Wistar rats (90–100 g) with a modified method originally described by Pittenger et al. [1999]. Briefly, bone marrow mononuclear cells were obtained by Percoll (1.073 g/ml) density gradient centrifugation. The cells were seeded in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) supplemented with 20% fetal bovine serum (FBS) and penicillin (100 U/ml) at 37°C in humified air with 5% CO_2. At 24 h after plating, nonadherent cells were removed by replacing medium. The antibiotic was removed after one media change. The medium was changed every 2–3 days and the cells were passaged in 0.05% trypsin-1 mM EDTA.

Flow Cytometric Analysis

Rat MSCs were phenotypically characterized by flow cytometry (Becton-Dickinson, San Jose, CA) by the method of Li et al. [2005]. The antibodies used in this study included FITC-conjugated or PE-conjugated antibodies CD14, CD29, CD34, CD44, CD45, CD105, HLA-ABC, HLA-DR. To detect surface antigens, cells were collected and incubated (30 min at 4°C) with the

respective antibody at a concentration previously established by titration. At least 1×10^5 cells for each sample were acquired and analyzed.

Cell Differentiation Induction

When the cultures of MSCs reached subconfluence, cells were washed twice with the medium and divided into two groups. In control group, the cells were cultured in basal DMEM medium (without FBS); in D609 treatment group, the cells were incubated with 5, 10, 15 μg/ml D609 in basal medium. Fresh D609 was dissolved in water and applied to cells. The cells in the two groups were incubated for 3 days. The morphological changes of the cells were observed under phase contrast microscope (Nikon, Japan).

Cell Viability Assay

Cells were seeded into 96-well plates and treated with or without D609 for 6, 48, 72 h, respectively. The viability of cells determined by using the method of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium) (Sigma Co.) assay as described previously [Price and McMillan, 1990]. The light absorption was measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI co.). The viability (%) was calculated by the formula as follow. Viability (%) = (OD of control or treated group/OD of normal group) × 100. The viability of normal group was presumed as 100%.

Immunocytochemistry and Immunofluorescence Assay

Immunocytochemistry and Immunofluorescence assay were performed as described previously [Wang et al., 2004]. After treatment with D609 for 6 h, cells were fixed in 4% paraformaldehyde for 15 min, blocked with normal goat serum for 20 min at room temperature (RT). Then, primary antibodies (rabbit anti-rat NSE, NF, synapsin, and Rb IgG) were added and incubated in a humid chamber over night. After washing with 0.1 M phosphate-buffered saline (PBS) three times, cells were incubated with appropriate secondary antibodies (FITC-goat anti-rabbit IgG) for 30 min at 37°C. After washing with 0.1 M PBS, the samples were evaluated under laser scanning confocal microscope (Leica, Germany). The immunofluorescence techniques allow semiquantitative evaluations of Rb protein expressions. The contents of Rb protein were showed as relative fluorescent intensity per cell.

The staining for neuronal specific marker NSE was used to estimate the differentiation rate of MSCs. The differentiation rate of MSC was calculated by the formula as follow. The differentiation rate (%) = (the number of positively stained cells/the total number of cells) × 100. Two hundreds cells for each sample were counted at least in random visual fields. The results presented are the mean ± SE derived from three independent experiments.

Analysis of PC-PLC Activity

The activity of PC-PLC in the cells was determined by the method published previously [Zhao et al., 2005]. In brief, we prepared the enzyme and used L-α-phosphatidylcholine as the substrate of PC-PLC. The optical density was measured at 660 nm (wavelength). Enzyme activity was expressed as nmol per min per mg of protein (nmol/min · mg).

Determination of Intracellular ROS Levels

Intracellular ROS levels were measured using a fluorescent probe, DCHF, which could be rapidly oxidized into the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. This assay is a reliable method for the measurement of intracellular ROS [Suematsu et al., 2003]. The fluorescence was monitored using excitation and emission wavelengths of 485 and 530 nm, respectively. The amount of ROS was quantified as the relative fluorescence intensity of DCF per cell in the scan room. The ROI (region of interest) was randomly selected, then zoomed in same folders, the value of relative fluorescent intensity per cell equals to the total value of sample in scan zoom divided by the total number of cells (at least 200 cells) in the same zoom. This assay repeated three times. Here, a representative result from the three similar experiments was shown.

Cytochrome c Reduction Assay

NADPH oxidase activity (NADPH-dependent O_2^- production) in cell homogenates was examined by using SOD-inhibitable cytochrome c reduction assay as described previously [Li et al., 2002]. Cell homogenate (final concentration 1 mg/ml) diluted in DMEM without phenol red was distributed in 96-well flat-bottom culture plates (final volume 200 μl/well). Cytochrome c (500 μmol/L) and NADPH

(100 μmol/L) were added in the presence or absence of SOD (200 U/ml) and incubated at RT for 30 min. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader. O_2^- production in nmol/mg protein was calculated from the difference between absorbance with or without SOD and the extinction coefficient for change of ferricytochrome c to ferrocytochrome c, that is, 21.0 mmol/L · cm.

SOD Activity Assay

The enzyme activity of intracellular SOD was detected in the MSCs treated with/without D609 for 6 h by using SOD detection kit. The activities of Manganese-dependent SOD (MnSOD) and Copper/Zinc-dependent SOD (Cu/ZnSOD) in cell homogenates were assessed according to the instructions provided by SOD detection kit. The optical density was measured at 550 nm (wavelength). The enzyme activity was expressed as U/mg of protein (U/mg protein).

Western Immunoblot Analysis

Cells were cultured in the absence or presence of D609 for 6 h. The total protein of the cells was prepared as described by Lipscomb E.A. [Lipscomb et al., 2003]. The concentration of each protein lysate was determined by the Bradford Protein Assay [Bradford, 1976]. Equal amount of total protein was loaded on 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose transfer membrane. After blocking with 5% skim milk in PBS, 0.5% (v/v) Tween-20 for 1 h, the membrane was incubated with polyclonal Rb protein antibodies (rabbit anti rat) overnight at 4°C, then incubated with HRP-linked secondary antibodies (goat anti rabbit) for 1 h at RT, followed by color development with 0.06% diaminobenzidine (DAB) and 0.03% H_2O_2 in PBS for 3–5 min. Distilled water was used to cease the reaction. Polyclonal anti-actin antibody (rabbit anti rat) was used to ascertain that equal amount of protein was loaded. The relative quantity of protein was analyzed by Imagetool software.

Statistical Analysis

Data were expressed as mean ± SE and accompanied by the number of experiments performed independently, and analyzed by *t*-test. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Immunophenotypic Characterization of Rat MSCs

Rat MSCs isolated in this study were uniformly positive for CD29, CD44, CD105, HLA-ABC (MHC-I molecule). In contrast, these cells were negative for other markers of the hematopoietic lineage CD14, CD34, the leukocyte common antigen CD45, and HLA-DR (MHC-II molecule) (Supporting Information). Flow cytometry analyses showed that the MSC was a homogeneous cell population devoid of hematopoietic cells.

D609 Induced the Neuronal Differentiation of Rat MSCs

Rat MSCs were exposed to 5, 10, 15 μg/ml D609 respectively in the absence of growth factors and serum. Recently, it is reported that, within 2 h, neural stem cells (NSCs) can convert to the vascular endothelial cells [Wurmser et al., 2004]. Based on this report, we observed the morphological changes of MSCs treated with D609 and examined the expressions of neuronal markers at 6 h. Responsive cells progressively assumed neuronal morphological traits. Initially, cytoplasm in the flat MSCs retracted towards the nucleus, forming a contracted multipolar cell body. Then the cell bodies became increasingly spherical and refractile. The processes formed extensive networks. At the same time, few cells fell off and died. Over the subsequent 3 days, the MSC-derived cells displayed typical neuronal morphology, ranging from simple bipolar cells to large, extensively branched multipolar cells. Meanwhile, more cells fell off and died. There were no obvious morphological changes in control group (Fig. 1A). When the cells were exposed to D609, the cells exhibited a typical neuronal appearance at 6, 48 and 72 h (Fig. 1B–D). The effect of D609 on MSC differentiation was in a dose-dependent manner and cell death happened during this process (Figs. 2 and 3). Under this serum-free condition, cell viabilities in the control and D609 treatment groups were decreased at 6, 48, and 72 h. The other reasons may be that cell death during normal nerve system development is a basic biological phenomenon and the neurite extension needs more space. D609 could induce neuronal differentiation of MSCs in a dose-dependent manner. There was a significant difference between

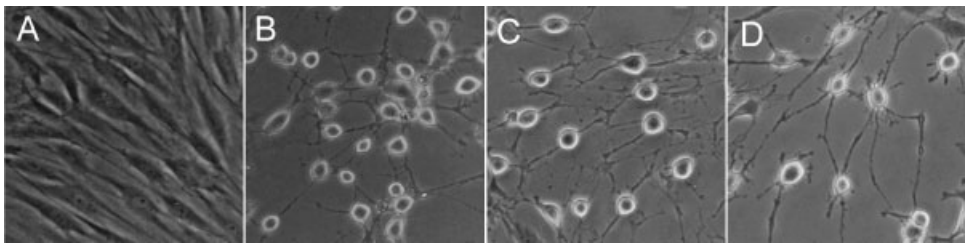

Fig. 1. The neuronal differentiation of rat MSCs treated with D609. **A**: The cells cultured in the basal medium. **B**–**D**: The morphological changes of cells treated with 10 μg/ml D609 at 6, 48, and 72 h, respectively (×200).

control group and each experiment group. The differentiation rate of MSCs gradually increased with the concentration of D609 in experiment groups ($P < 0.01$). Thus we chose 10 μg/ml D609 as the most proper concentration (this concentration similar to that used to inhibit PC-PLC in other cells) [Li et al., 1998; Andrei et al., 2004].

To confirm the characters of these morphologically changed MSCs, the expressions of NSE, NF-L, and synapsin were examined. Immunocytochemistry experiment results showed that at 6 h, the MSCs treated with D609 displayed weak expressions of NSE, NF-L, and synapsin (Fig. 4). At 72 h, in control groups, the undifferentiated flat cells showed very weak expressions of NSE, NF-L, and almost no specific staining for synapsin, whereas in D609 treatment groups, the differentiated spherical cells exhibited intensive positive NSE, NF-L, and synapsin (Fig. 4). These results showed that D609 could induce neuronal differentiation in rat MSCs. The expression of synapsin further showed that MSCs-derived neuronal phenotypic cells possessed some characteristics of real neurons. We found that almost all of the MSCs with a typical neuronal appearance displayed intensive positive NSE and NF-L.

PC-PLC Activity was Inhibited by D609 During the Neuronal Differentiation of Rat MSCs

To investigate the underlying mechanism of the phenomenon mentioned above, we examined the activity of PC-PLC in the cells treated and untreated with D609. Our results showed that there were at least two isoforms of PC-PLC

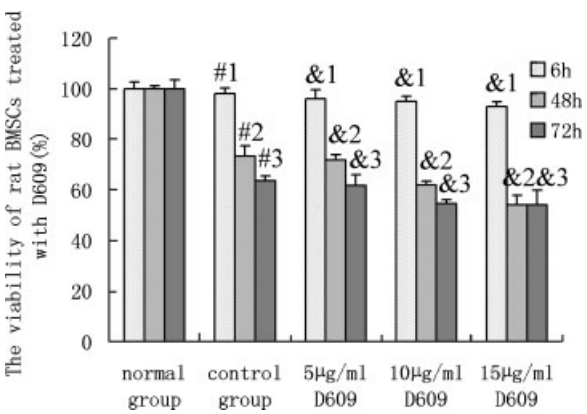

Fig. 2. The viability of rat MSCs treated with D609. Normal group, the viability of the cells cultured in DMEM medium with FBS for 6, 48, 72 h, respectively; control group, the viability of the cells cultured in basal DMEM medium (without FBS) for 6, 48, 72 h, respectively; D609 treatment group, the viability of the cells treated with 5, 10, 15 μg/ml D609 in basal medium for 6, 48, 72 h, respectively. The cell viability gradually decreased with the treatment time both in control groups and in experiment groups. There were no differences between control group and each experiment group; ($P > 0.05$) $^{\&}P > 0.05$ versus #, n = 3.

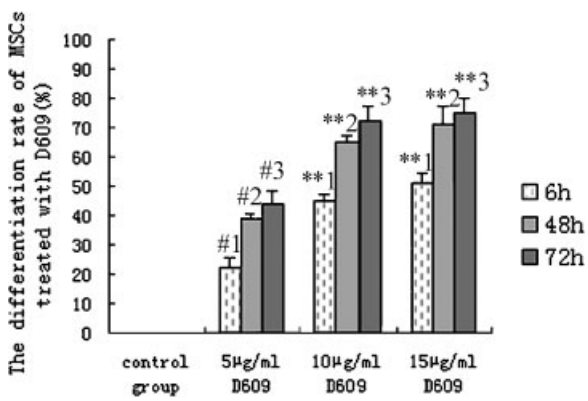

Fig. 3. The differentiation rate of rat MSCs treated with 0, 5, 10, 15 μg/ml D609 for 6, 48, 72 h, respectively. The differentiation rate of MSCs was estimated by calculating the frequency of the cells that were positively stained for NSE. D609 could induce neuronal differentiation of MSCs in a dose-dependent manner. There was a significant difference between control group and each experiment group. The differentiation rate of MSCs gradually increased with the concentration of D609 in experiment groups; ($P < 0.01$) $^{**}P < 0.01$ versus #, n = 3.

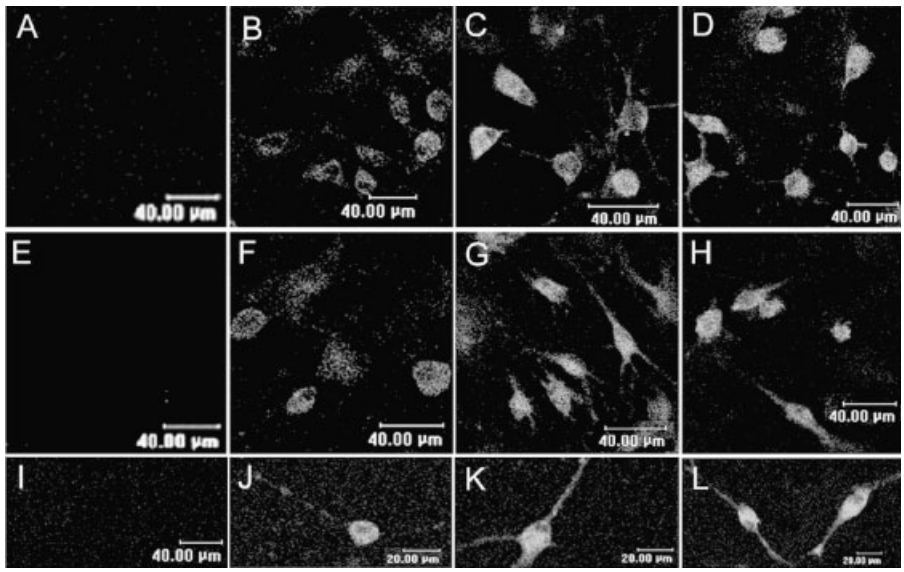

Fig. 4. Expression of mature neural proteins in rat MSCs treated with 10 μg/ml D609. **A**: The cells in control group showed weak expression of NSE at 72 h. **B**–**D**: The cells treated with D609 exhibited NSE intensive positive at 6, 48, and 72 h, respectively. **E**: The cells in control group showed weak expression of NF at 72 h. **F**–**H**: The cells treated with D609 exhibited NF intensive positive at 6, 48, and 72 h, respectively. **I**: The cells in control group showed almost no specific staining for synapsin at 72 h. **J**–**L**: The cells treated with D609 exhibited synapsin intensive positive at 6, 48, and 72 h, respectively. The experiment was repeated three times with similar results.

in rat MSCs. They were the Ca^{2+}-dependent PC-PLC and the Ca^{2+}-independent PC-PLC. As shown in Figure 5, following the treatment with D609, the activities of the both PC-PLC decreased significantly ($P < 0.05$). These data showed that D609 actually inhibited the activity of PC-PLC during the neuronal differentiation of rat MSCs.

The Level of Intracellular ROS was Elevated During the Neuronal Differentiation of Rat MSCs

To understand whether ROS are implicated in the neural differentiation of rat MSCs, we detected the levels of intracellular ROS in the cells treated and untreated with D609. As shown in Figure 6A–C, in the undifferentiated MSCs, the relative fluorescent intensity of DCF was low, but in the differentiated MSCs, the fluorescent intensity increased significantly ($P < 0.05$). Our results showed that the intracellular ROS level was elevated obviously during the neuronal differentiation of rat MSCs. D609 at the concentrations of 5–15 μg/ml could not depress intracellular ROS level when it induced neuronal differentiation of rat MSC.

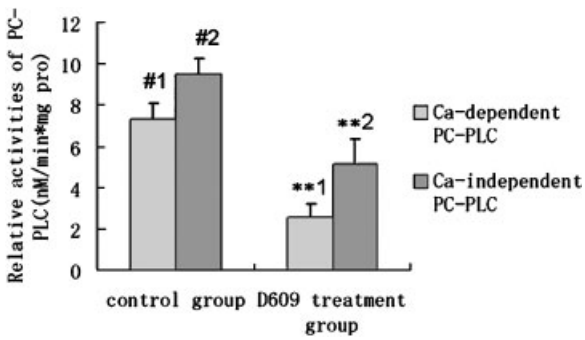

Fig. 5. PC-PLC activity was inhibited by D609 during neuronal differentiation of rat MSCs. The activities of the two PC-PLC isoforms decreased markedly in the cells treated with 10 μg/ml D609 at 6 h; ($P < 0.01$) $^{**}P < 0.01$ versus #, n = 3.

NADPH Oxidase was Activated During the Neuronal Differentiation of Rat MSCs

To understand which enzymes participate in the regulation of ROS level in the neuronal differentiation of rat MSCs, we investigated the changes of NADPH oxidase and SOD activities. As shown in Figure 6D, when MSCs were induced differentiation into neuron-like cells, the activity of NADPH oxidase increased significantly ($P < 0.01$). At the same time, compared with control group, Cu/ZnSOD and MnSOD activities in D609 treatment group were not remarkably altered (Fig. 6E, $P > 0.05$). These data showed that NADPH oxidase-derived ROS might be involved in the MSC differentiation mediated by PC-PLC.

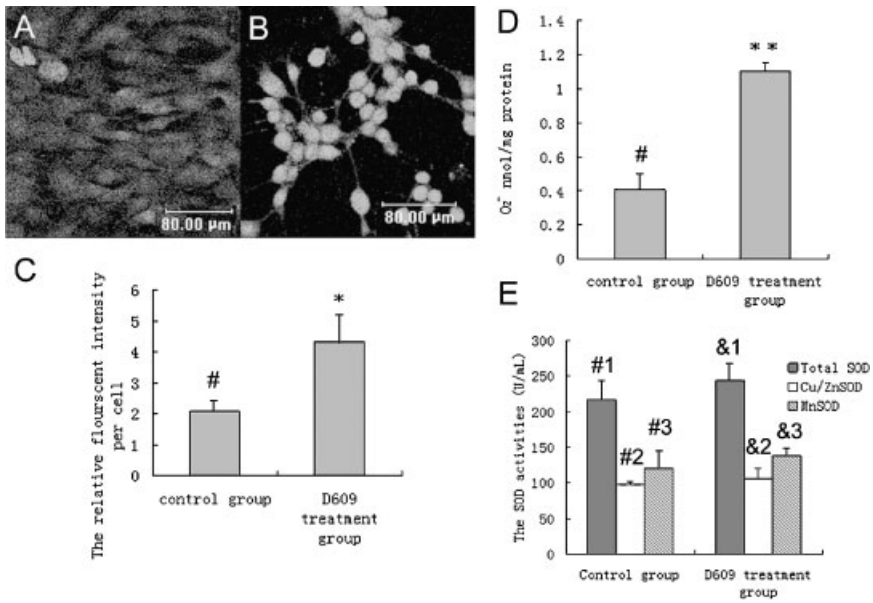

Fig. 6. The changes of intracellular ROS level, NADPH oxidase and SOD activities during neuronal differentiation of rat MSCs. Fluorescent micrographs show the relative intensity of ROS. **A**: The cells cultured in the basal medium for 6 h. **B**: The cells treated with 10 μg/ml D609. **C**: The quantity of intracellular ROS levels; ($P < 0.05$) $^{*}P < 0.05$ versus #, n = 3. **D**: The changes of NADPH oxidase activity during neuronal differentiation of rat MSC; ($P < 0.01$) $^{**}P < 0.01$ versus #, n = 3. **E**: The changes of SOD activities during neuronal differentiation of rat MSC. Compared with control group, Cu/ZnSOD and MnSOD activities in D609 treatment group were not obviously altered; ($P > 0.05$) $^{\&}P > 0.05$ versus #, n = 3.

Inhibiting PC-PLC Increased the Expression of Rb Protein During the Neuronal Differentiation of Rat MSCs

To investigate whether Rb protein is implicated in the regulation of MSC differentiation mediated by PC-PLC, we detected the expression of Rb protein by Immunofluorescence and Western blot analysis. It was observed that the Rb level was increased during the neuronal differentiation of rat MSCs (Fig. 7, $P < 0.05$). This result showed that Rb protein might contribute to the neuronal differentiation of MSCs mediated by PC-PLC.

DISCUSSION

In previous reports, several inducers must be used to induce MSC differentiation to neuron [Woodbury et al., 2000, 2002]. Under these complex conditions, it is difficult to understand the mechanism by which MSCs differentiate to neurons. In this study, we found that PC-PLC had an important role in controlling neural differentiation of MSCs. This finding leads us to investigate the possible mechanism by which PC-PLC regulates the neuronal differentiation of MSCs.

Previously, we demonstrated that D609 could induce some hVECs and hMSCs to differentiate into neuron-like cells [Wang et al., 2004]. In this study, the data provided the evidence that D609 could also induce neuronal differentiation of rat MSCs. These results suggested that the role of D609 was universal to some extent.

It has been reported that the differentiation of MSCs into neuronal phenotypes required the administration of growth factors [Bossolasco et al., 2005; Long et al., 2005; Tao et al., 2005]. In this study, our results provided the new evidence that MSCs could be induced to differentiation in the absence of growth factors. The data indicated that a new signal pathway in which PC-PLC was implicated might be activated during the neuronal differentiation of MSCs.

D609 has been shown to inhibit bFGF-stimulated cell proliferation by the way of inhibiting PC-PLC and sphingomyelin biosynthesis in primary astrocytes [Riboni et al., 2001]. Recent experiment results showed that the PC-PLC pathway acted as a novel pathway downstream of the FGF receptors in retinal ganglion cells [Webber et al., 2005]. The activities of Ca^{2+}-dependent PC-PLC decreased significantly during RA-induced differentiation

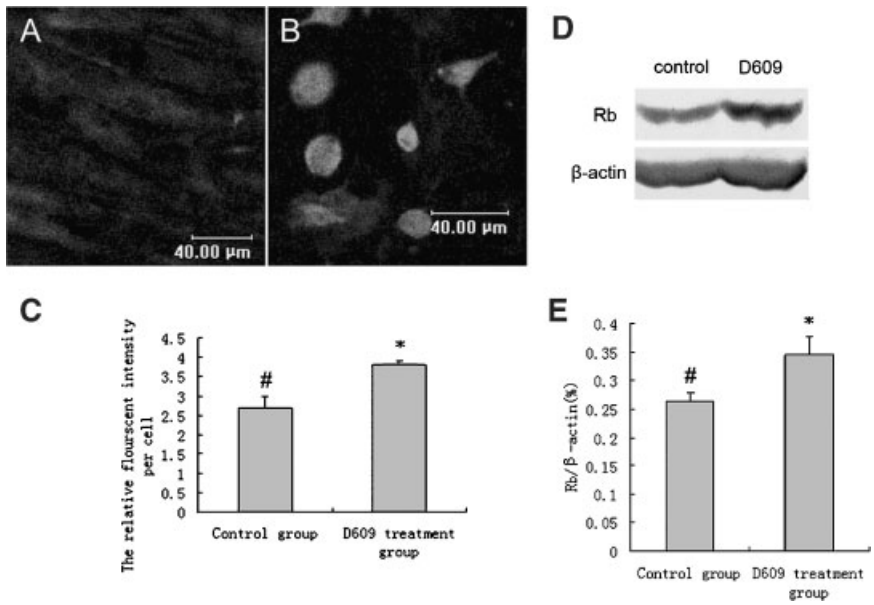

Fig. 7. The expression of Rb protein increased during neuronal differentiation of rat MSCs. Fluorescent micrographs show the relative intensity of Rb. **A**: The cells cultured in the basal medium for 6 h. **B**: The cells treated with 10 μg/ml D609. **C**: The quantity of expressed Rb protein. ($P < 0.05$) $^*P < 0.05$ versus #, n = 3. **D**: The bands of proteins by Western blot assay. **E**: The relative quantity of Rb protein depicted as a bar chart; ($P < 0.05$) $^*P < 0.05$ versus #, n = 3.

of CBRH-7919 cells [Wu et al., 1997]. In Friend leukemia cells, PC-PLC activation is involved in erythroid differentiation [Ferretti et al., 1993]. In macrophages, the differentiation signal relies on PKC and PC-PLC activation [Buscher et al., 1995]. Our data further suggested the importance of PC-PLC in cell differentiation. But, during the neuronal differentiation of MSCs, how PC-PLC works is not known.

Several studies showed that nontoxic level of ROS could play an essential role as signal molecules in regulating cell growth and differentiation. In addition, ROS were recently suggested as inducers of neuronal differentiation [Peunova and Enikolopov, 1995; Kamata et al., 1996]. Differentiation of PC12 cells in response to nerve growth factor requires ROS production [Katoh et al., 1997; Suzukawa et al., 2000]. Our results showed that D609 could not depress intracellular ROS level in the MSCs. On the one hand, the data suggested that the neuronal differentiation of MSCs might require high level of intracellular ROS. On the other hand, our data indicated that the antioxidant D609 at the low concentrations was not enough to depress the high ROS level induced by the cell differentiation. Taken together with other studies [Brookes et al., 2002; Finkel, 2003], our findings are consistent with the idea that ROS may serve as important signal molecules in the neuronal differentiation of MSCs.

A critical balance between the synthesis and destruction of ROS is likely very important for the regulation of cell fate, including proliferation, differentiation and death. There are at least two pivotal enzymes involved in the regulation of ROS production, NADPH oxidase and SOD. ROS derived from NADPH oxidase have been reported to be involved in NGF-induced differentiation in PC12 cells [Suzukawa et al., 2000]. SOD is one efficient anti-oxidant enzyme in BMSCs [Ebert et al., 2006]. In this study, we found that the activity of NADPH oxidase was elevated significantly, but MnSOD and Cu/ZnSOD activities were not changed during the neuronal differentiation of MSCs. The data suggested that in this differentiation process the increased NADPH oxidase activity might result in the elevating of ROS level. Taken together with previous experimental results [Suzukawa et al., 2000], we supposed that NADPH oxidase-derived ROS might play an important role in PC-PLC-mediated MSC differentiation signal transduction pathways.

Numerous studies implicate the Rb protein as a key regulator of terminal differentiation in the

developing central nerve system (CNS). For example, Rb is required for terminal differentiation of cerebellar granule cells [Marino et al., 2003] and keratinocytes [Ruiz et al., 2004]. Furthermore, there is a critical temporal requirement for the Rb genes during neuronal differentiation of MSCs [Jori et al., 2004, 2005a]. Our findings are consistent with the idea that Rb protein is highly up-regulated during the process of neuronal differentiation in MSCs. The data in this study indicated that PC-PLC and Rb protein might be in the same signal transduction pathway during the MSC differentiation induced by D609.

In summary, our results in this study showed that the role of D609 in inducing MSC neuronal differentiation was universal to some extent. When D609 induced neuronal differentiation of rat MSCs, the expression of Rb protein was increased simultaneously with the suppression of PC-PLC activity. Intracellular ROS level and NADPH oxidase activity in the differentiated MSCs were significantly higher than that in the undifferentiated MSCs. The data indicated that PC-PLC mediated the differentiation signaling by up-regulating the expression of Rb protein. During the neuronal differentiation of MSCs, ROS that were controlled probably by NADPH oxidase might serve as important signal molecules.

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