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Inhibition of Phosphatidylcholine-Specific Phospholipase C Prevents Bone Marrow Stromal Cell Senescence In Vitro

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ABSTRACT

Bone marrow stromal cells (BMSCs) can proliferate in vitro and can be transplanted for treating many kinds of diseases. However, BMSCs become senescent with long-term culture, which inhibits their application. To understand the mechanism underlying the senescence, we investigated the activity of phosphatidylcholine-specific phospholipase C (PC-PLC) and levels of integrin β4, caveolin-1 and ROS with BMSC senescence. The activity of PC-PLC and levels of integrin β4, caveolin-1 and ROS increased greatly during cell senescence. Selective inhibition of increased PC-PLC activity with D609 significantly decreased the number of senescence-associated beta galactosidase positive cells in BMSCs. Furthermore, D609 restored proliferation of BMSCs and their differentiation into adipocytes. Moreover, D609 suppressed the elevated levels of integrin β4, caveolin-1 and ROS. The data suggest that PC-PLC is involved in senescence of BMSCs, and its function is associated with integrin β4, caveolin-1 and ROS. J. Cell. Biochem. 108: 519–528, 2009.

KEY WORDS: BONE MARROW STROMAL CELLS; SENESCENCE; PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C; INTEGRIN β4; CAVEOLIN-1; ROS

The discovery of adult stem cells has allowed for generating new tissue and repairing tissue damage. As one kind of adult stem cells, bone marrow stromal cells (BMSCs) have drawn great attention for their low immunogenicity and ability to differentiate into various kinds of cells. However, the proportion of BMSCs in the bone marrow is very small, with an estimated frequency in mice of only one in $1–3 \times 10^4$ cells [Beausejour, 2007]. Therefore, in vitro expansion protocols are needed to increase the number of BMSCs for transplantation.

When the cells are cultured in vitro, their proliferation ability diminishes and their functions alter with increased cell passage. Ultimately, cells undergo senescence to reach the Hayflick limit [Hayflick, 1965]. Accumulating evidence has demonstrated that the ability to differentiate into osteoblasts and adipocytes is lower in senescent BMSCs than in young BMSCs [Sethe et al., 2006]. Therefore, the application of BMSCs in the clinic is affected by their aging. Understanding the mechanism of BMSC senescence is crucial.

Phosphatidylcholine-specific phospholipase C (PC-PLC), a member of phospholipase C family, catalyzes the hydrolysis of the ester linkage between glycerol and phosphate in phosphocholine [Szumilo and Rahden-Staron, 2008]. The hydrolysis products phosphocholine and diacylglycerol are second messengers implicated in a wide range of cellular responses. Much evidence has suggested a role for PC-PLC in metabolism, growth, differentiation and apoptosis of mammalian cells [Miao et al., 1997b; Zhao et al., 2007; Wang et al., 2007a, 2008; Spadaro et al., 2008]. In previous research, we found the activity of PC-PLC altered with senescence of vascular endothelial cells (VECs) [Liu et al., 2007b]. PC-PLC is important in mitogen-stimulated fibroblasts, and inhibition of its activity by D609 resulted in a G_0-arrest [Ramoni et al., 2004]. However, whether PC-PLC participates in the senescence of BMSCs is unknown.

Integrin β4 has an exceptionally large cytoplasmic domain and is associated with cytoskeletal and signaling molecules [Hogervorst et al., 1990]. It has important roles in cellular signal transduction [Bertotti et al., 2006; Su et al., 2008]. We have studied the roles of integrin β4 in VEC senescence and apoptosis and in neural stem cell differentiation and neuron survival; during VEC senescence and

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apoptosis, the level of integrin β4 was increased, but with decreased expression of integrin β4, senescence and apoptosis is suppressed [Liu et al., 2007b; Miao et al., 1997a; Wang et al., 2007b]. Recently, we found that knockdown of integrin β4 inhibited mouse neural stem cell differentiation, whereas overexpression of integrin β4 promoted the differentiation [Su et al., 2009]. Downregulation of integrin β4 by its siRNA in primary culture mouse neurons produced apoptosis in neurons. The level of reactive oxygen species (ROS) in neurons was elevated significantly as was the activity of NADPH oxidase, but activities of manganese-dependent SOD and copper/zinc-dependent SOD were not altered. However, the functions of PC-PLC and integrin β4 are known to be cell specific [Raymond et al., 2007], and whether integrin β4 is associated with PC-PLC activity during BMSC senescence is unknown.

Senescent human diploid fibroblasts showed upregulated caveolin-1 protein [Park et al., 2000]. As well, caveolin-1 can induce premature cellular senescence in primary cultures of murine fibroblasts [Volonte et al., 2002] and in human diploid fibroblasts [Chretien et al., 2008]. Upregulation of caveolin-1 might accelerate the senescence while impairing the proliferation of cells. As well, overexpressed caveolin-1 in young human BMSC could suppress both insulin signaling and adipogenic differentiation by down-regulated PPARgamma2 [Park et al., 2005]. However, the relation between caveolin-1 and PC-PLC in BMSCs is not known.

Accumulation of ROS in cells is an indicator and a cause of aging [Young et al., 2001]. ROS is harmful to BMSC-dependent regeneration. Increased ROS production resulted in reduced glutathione peroxidase and alkaline phosphatase activities in young females, which was similar to old females rats [Isomura et al., 2004]. As well, ROS is involved in the senescence of rat BMSCs [Stolzing et al., 2006]. However, the relation between ROS and PC-PLC is not understood in BMSC senescence.

To address these questions, we investigated the changes of PC-PLC and its association with integrin β4, caveolin-1, and ROS during senescence of cultured BMSCs.

MATERIALS AND METHODS

CELL CULTURE

Rat BMSCs were isolated from the femurs and tibias of male Wistar rats (90–100 g) as described [Pittenger et al., 1999]. The cells were seeded in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Gibco, USA) supplemented with 10% FBS (Hyclone, USA) at 37°C in humified air with 5% CO_2. Rat BMSCs were phenotypically characterized as described [Wang et al., 2007a]. The experiments were performed on cells with a population doubling level (PDL) of 2–8. The PDL is calculated for a passage increase according to the following equation: $\log_2$ {(number of collected cells)/(number of seeded cells)} [Tanaka et al., 2007].

CELL TREATMENT

To detect the role of PC-PLC in senescence of rat BMSCs, we treated senescent cells with D609 to inhibit the activity of PC-PLC. When cells reached sub-confluence, 4, 8, and 16 μg/ml D609 dissolved in DMEM-LG supplemented with 10% FBS was added to the cells for 24-h incubation.

Fresh D609 was dissolved in water. Morphological changes were observed on phase-contrast microscopy (Nikon, Japan).

SENESCENCE-ASSOCIATED BETA-GALACTOSIDASE (SA-β-GAL) STAINING

Because no true molecular marker of cellular senescence exists [Cichowski and Hahn, 2008], BMSC senescence can be identified by SA-β-Gal staining [Chen and Goligorsky, 2006]. In brief, cells were rinsed twice with phosphate-buffered saline (PBS), fixed for 5 min in formaldehyde, and rinsed with PBS. An amount of 2 ml staining solution was added to each 35-mm dish. After overnight incubation at 37°C, the staining solution was removed. The cells were rinsed with PBS and observed on phase-contrast microscopy. Senescent cells were stained blue. The proportion of positively stained cells was estimated by counting cells in random visual fields, with at least 2,000 cells counted for each sample.

PROLIFERATION ASSAY

BMSCs at PDL 2 or 8 were suspended in DMEM-LG with 10% FBS and seeded in 35-mm dishes at 2×10^4 cells. A group of PDL 8 cells was treated with 4 μg/ml D609 for 8 days. The number of cells was determined by use of a hemacytometer at days 2, 4, 6, and 8 after treatment with 0.05% trypsin–EDTA.

ADIPOGENIC DIFFERENTIATION OF BMSCS

The differentiation of BMSCs to adipocytes was examined as described [Pittenger et al., 1999]. When cells reached confluence in 6-well plates, the growth medium was replaced with DMEM-LG containing 1 μM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.01 mg/ml insulin and 10% FBS. The induction medium was changed every 2 days for 10 days. Morphological changes were observed on phase-contrast microscopy.

OIL-RED O STAINING

Oil-red O staining, an indicator of intracellular lipid accumulation, can be used to assess adipogenic differentiation of BMSCs. After BMSC differentiation to adipocytes, cells were rinsed twice with PBS, fixed with 10% formalin in PBS (pH 7.4) for 10 min, then washed with 100% isopropanol to dehydrate. BMSCs were stained with 0.3% Oil-red O for 10 min at 60°C. After three washes with PBS, cells were visualized on light microscopy and photographed. As well, the cell number was counted in random visual fields. To extract incorporated Oil-red O, 1 ml 100% isopropanol was added to each well, which was followed by 15-min shaking at room temperature. After appropriate dilution, samples were read at 510 nm. The Oil-red O staining per cell was calculated as follows: (ΔAsample − ΔAblank)/cell number. ΔA = change in absorbance.

IMMUNOFLUORESCENCE ASSAY

Immunofluorescence assay was performed as described [Wang et al., 2007a]. Cells were fixed in 4% paraformaldehyde (w/v) for 20 min at room temperature and blocked in goat serum. After two rinses with 0.1× PBS, cells were incubated with primary antibodies (rabbit polyclonal anti-integrin beta4/caveolin-1 IgG) (Santa Cruz, USA) overnight at 4°C. Then cells were washed with 0.1× PBS 3 times and

appropriate secondary antibodies (FITC-goat anti-rabbit IgG) were added for 1 h at 37°C. Samples were evaluated on laser scanning confocal microscopy (Leica, Germany). The relative intensity per cell was calculated as [(intensity of cells − intensity of background) × area of cells]/total number of cells in random visual fields. At least 200 cells were counted for each sample.

PC-PLC ACTIVITY ASSAY

PC-PLC activity assay was performed as described [Wu et al., 1997]. Briefly, the reaction mixture (500 μl) contained 100 mmol/L Tris–HCl, pH 7.0, 1 mmol/L EDTA, 1.5 mmol/L $CaCl_2$, and 5 mmol/L L-α-PC. The reaction was started by adding 50 μl of the sample enzyme to the mixture, which was included in the total volume. The tubes with 50 μl water were control tubes. After incubation at 37°C for 15 min, the reaction was stopped by adding 2 ml chloroform/methanol/HCl (2:1:0.035, v/v/v). The reaction mixture was then vigorously agitated in a vortex mixer and centrifuged at 1,000*g* for 10 min. Each supernatant (160 μl) was transferred to another mixture (500 μl) containing 0.21 mol/L Tris, pH 9.8, 50 mmol/L $MgCl_2$, and 4 units of alkaline phosphatase. After incubation at 37°C for 30 min, 2 ml of chromogen solution (0.31% ammonium molybdate, 0.66 mol/L sulfuric acid, 0.011% malachite green, and 0.018% Tween-20) was added, and the resulting mixture was incubated at 37°C for another 20 min. The optical density was measured at 660 nm (wavelength) by use of a SpectraMAX 190 microplate spectrophotometer (GMI Co., USA). PC-PLC activity was calculated as follows:

$$\text{PC} - \text{PLC activity} = (\Delta\text{Asample/min} - \Delta\text{Ablank/min}) \times \text{F}$$

where F = V total/(V sample × sample protein concentration); ΔA = change in absorbance; V = volume in ml.

DETECTION OF INTRACELLULAR ROS LEVEL

Intracellular ROS levels were measured by use of a fluorescent probe, DCHF, which can 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 at 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 zoomed field.

WESTERN BLOT ANALYSIS

Western blot and relative protein quantity analysis were performed as described [Du et al., 2005]. In brief, cells under various treatments were lysed in protein lysis buffer (1% SDS in 25 mM Tris–HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor). The protein concentration of cell was determined on Coomassie brilliant blue protein assay. BMSC protein extracts (30 μg) were applied to 9% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated with anti-caveolin-1, anti-integrin β4 or anti-β-actin antibodies (1:1,000 dilution) (Santa Cruz) and then detected with a horseradish peroxidase-conjugated IgG. Band intensity was quantified by use of Quantity one software (Bio-Rad, USA) and normalized to β-actin levels.

STATISTICAL ANALYSIS

All experiments were performed in duplicate and repeated three to five times. Data are expressed as means ± SE. Statistical analysis involved the Student *t*-test. A *P* of <0.05 was considered statistically significant (SPSS).

RESULTS

PC-PLC ACTIVITY INCREASED DURING BMSC SENESCENCE

The number of SA-β-gal positive cells in PDL 8 cells was higher than in PDL 2 cells (Fig. 1A). Thus, BMSCs became senescent after several passages, and PDL 2 and 8 cells could be adopted as young and senescent cells, respectively, in subsequent experiments. Mammal cells contain two kinds of PC-PLC: Ca^{2+}-dependent and -independent [Wu et al., 1997]. To determine whether and which kind of PC-PLC is involved in BMSC senescence, the activities of the PC-PLCs in BMSCs at different PDL were examined. Both

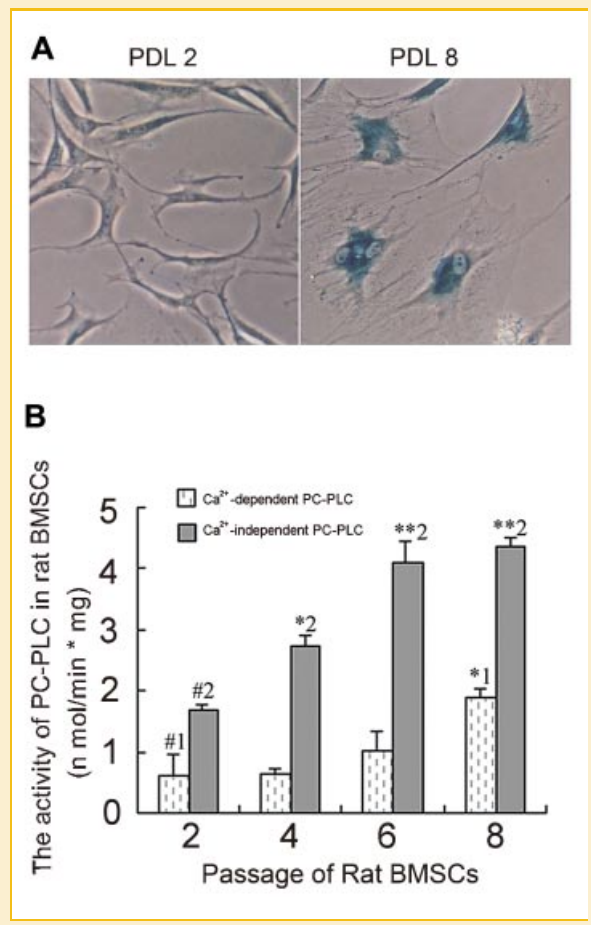

Fig. 1. Alteration in phosphatidylcholine-specific phospholipase C (PC-PLC) activity during bone marrow stromal cell (BMSC) senescence. A: Senescence-associated beta-galactosidase (SA-β-Gal) activity in BMSCs with population doubling level (PDL) 2 or 8. B: Ca^{2+}-dependent and -independent PC-PLC evaluated in BMSCs at PDL 2, 4, 6 and 8. *1, $P < 0.05$ versus #1; *2, $P < 0.05$ versus #2; **2, $P < 0.01$ versus #2, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

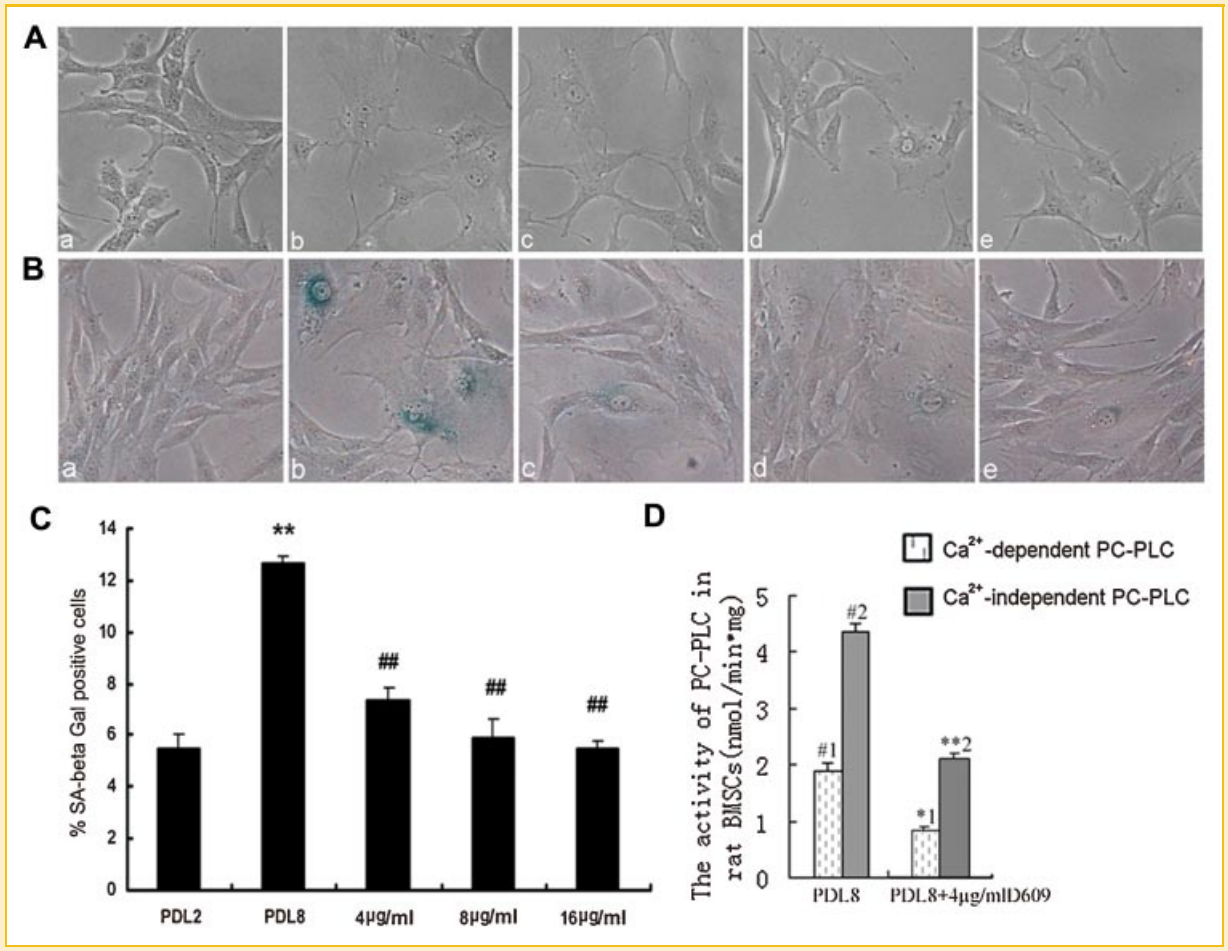

Fig. 2. Suppression of PC-PLC inhibited BMSC senescence. A: The morphology of young and senescent BMSCs. B: SA-β-Gal activity in BMSCs. C: Percentage of SA-β-Gal-positive cells. In (A) and (B), (a) and (b) represent the percentage of SA-β-Gal-positive cells in BMSCs at PDL 2 and 8, respectively. c–e: Percentages of SA-β-Gal-positive cells in BMSCs at PDL 8 treated with 4, 8, or 16 μg/ml D609 ($^{**}P < 0.01$ vs. PDL 2 cells; $^{\#\#}P < 0.01$ vs. PDL 8 cells, n = 4). D: 4 μg/ml D609 inhibited the activity of PC-PLC. $^{*}1$, $P < 0.05$ versus $^{\#}1$; $^{**}2$, $P < 0.01$ versus $^{\#}2$, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ca^{2+}-dependent and -independent PC-PLC was increased during cell senescence ($P < 0.05$ and $P < 0.01$, respectively; Fig. 1B).

SUPPRESSION OF PC-PLC ACTIVITY BY D609 INHIBITS BMSC SENESCENCE

To determine the involvement of PC-PLC in cell senescence, we treated senescent cells with D609, the selective inhibitor of PC-PLC activity. Young cells exhibited a typical spindle shape (Fig. 2Aa). After several passages, the number of fibroblast like cells decreased, and more cells flattened and became larger; as well, the cytoplast area and the ratio of cytoplasm to nucleus increased; more vacuoles appeared in cytoplasm (Fig. 2Ab). However, when senescent cells were treated with D609 at 4, 8, and 16 μg/ml, the number of flattened cells greatly decreased (Fig. 2Ac–e).

Compared with young cells, senescent BMSCs showed a significantly increased proportion of SA-β-Gal-positive cells ($P < 0.01$) (Fig. 2Bb,C). Treated with 4, 8, and 16 μg/ml D609 markedly decreased the number of SA-β-Gal-positive cells (Fig. 2Bc–e,C). As well, the

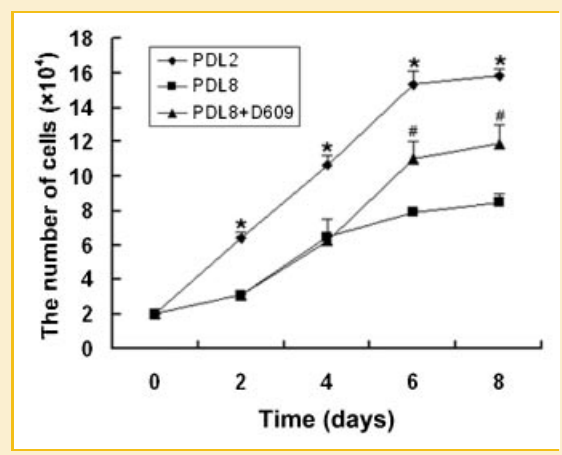

Fig. 3. Effect of D609 on proliferation of BMSCs. The growth rate of BMSCs at PDL 2 and 8 and PDL 8 with 4 μg/ml D609. $^{*}P < 0.05$; $^{\#}P < 0.05$ versus PDL 8 cells, n = 4.

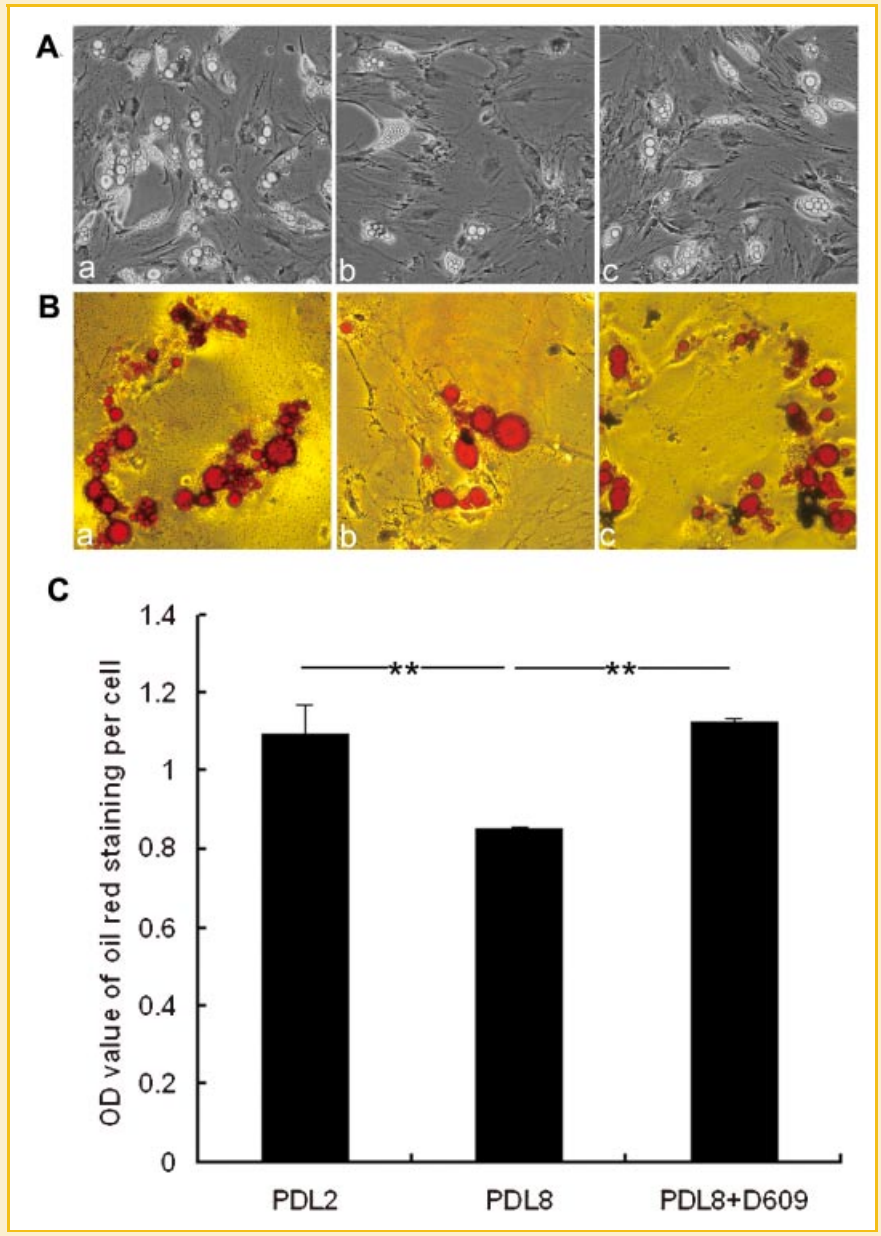

Fig. 4. The adipogenic differentiation of young and senescent BMSCs. A: Morphology of BMSCs at PDL 2 (a), PDL 8 (b) and PDL 8 treated with D609, 4 μg/ml (c). B: Oil-red O staining of BMSCs at PDL 2 (a) and 8 (b) and at PDL 8 with D609, 4 μg/ml (c). C: Relative quantity of Oil-red O staining per cell in the three groups. $^{**}P < 0.01$, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activity of Ca^{2+}-dependent and -independent PC-PLC in senescent cells was significantly inhibited by D609 at 4 μg/ml (Fig. 2D). Thus, inhibition of PC-PLC by D609 could inhibit the senescence of BMSCs. Thus, in subsequent experiments, we treated BMSCs with 4 μg/ml D609 to inhibit senescence.

One sign of in vitro aging is a diminished capacity for division. We identified the proliferative activity in BMSCs during senescence. Cell expansion was slower in PDL 8 cells than in PDL 2 cells, and the replicative ability was significantly elevated when senescent cells were incubated with 4 μg/ml D609 for 6 days (Fig. 3). In addition, we tested the capacity of senescent BMSCs to differentiate into adipocyte (Fig. 4). D609 inhibited the decreased differentiation to adipocyte caused by aging, as seen by Oil-red O staining.

INCREASED LEVEL OF INTEGRIN β4 IN SENESCENT BMSCS WAS INHIBITED BY D609

To determine whether the level and distribution of integrin β4 is altered in senescent BMSCs, we performed immunofluorescence assays of integrin β4 in young and senescent cells. Integrin β4 level in senescent BMSCs was significantly higher than that in young cells ($P < 0.05$) (Fig. 5B). Of interest, integrin β4 was distributed evenly in the membrane of young but not senescent cells (Fig. 5Ab,c). On

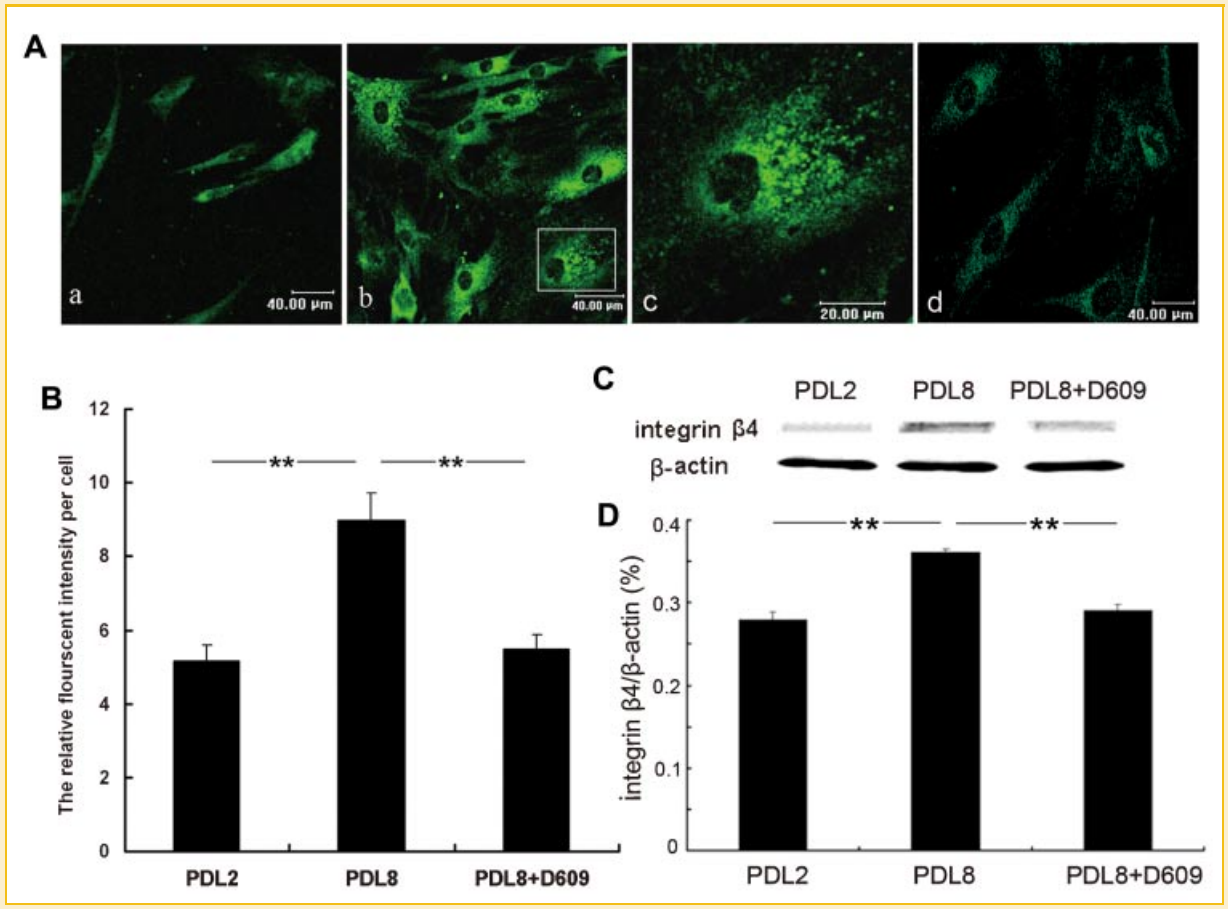

Fig. 5. Integrin β4 level during BMSC senescence. A: Fluorescent micrographs of relative intensity of integrin β4 in young and senescent BMSCs. Location and relative intensity of integrin β4 in BMSCs at (a) PDL 2 and (b) PDL 8. c: Magnification of b. d: Distribution and intensity of integrin β4 in BMSCs at PDL 8 with 4 μg/ml D609. B: Relative quantity of integrin β4 level. $^{**}P < 0.01$, n = 3. C: Western blot assay of integrin β4 protein level. D: Quantification of integrin β4 level shown in C. $^{**}P < 0.01$, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment with D609, 4 μg/ml, the level of integrin β4 decreased remarkably (Fig. 5B), and the aggregation of integrin β4 disappeared (Fig. 5Ad). The alteration in integrin β4 protein level was confirmed by Western blot analysis (Fig. 5C,D). Therefore, integrin β4 was associated with PC-PLC in BMSC senescence.

CAVEOLIN-1 LEVEL IS DECREASED IN D609-TREATED SENESCENT BMSCs

Caveolin-1 is a key factor in BMSC senescence [Park et al., 2005]. We found the level of caveolin-1 in the senescent BMSCs higher than that in young cells ((Fig. 6A,B, $P < 0.01$). We further examined the changes in caveolin-1 level on treatment of cells with D609, 4 μg/ml (Fig. 6Ac): D609 inhibited the increased level of caveolin-1(Fig. 6B). We further confirmed these changes by the Western blot analysis (Fig. 6C,D).

D609 INHIBITED THE INCREASED ROS LEVEL IN SENESCENT BMSCs

ROS level is known to be enhanced during BMSC senescence. To further demonstrate the inhibition of D609 on cell senescence and understand the relation of PC-PLC and ROS in the process, we determined the level of ROS in D609-treated senescent BMSCs. The level of ROS was decreased on inhibition of senescence of BMSCs by D609, 4 μg/ml (Fig. 7, $P < 0.05$).

DISCUSSION

BMSCs can differentiate into several kinds of cells and be used in treatment for a number of diseases. However, the aging of BMSCs results in their decreased ability to self-renew and properly differentiate [Roobrouck et al., 2008], which does not favor their use in the clinic. Hence, defining the mechanism underlying BMSC senescence is crucial. In the present study, the activity of PC-PLC and the levels of integrin β4, caveolin-1 and ROS were greatly increased during BMSC senescence. Inhibition of the increased PC-PLC activity by the specific PC-PLC inhibitor D609 in the senescent cells suppressed the cell senescence and decreased the number of SA-β-gal-positive senescent cells and restored their proliferation and differentiation into adipocytes. Moreover, the elevation of integrin β4, caveolin-1 and ROS levels in senescent BMSCs was greatly suppressed by D609. PC-PLC could be a key factor in the senescence of BMSCs and is associated with integrin β4, caveolin-1

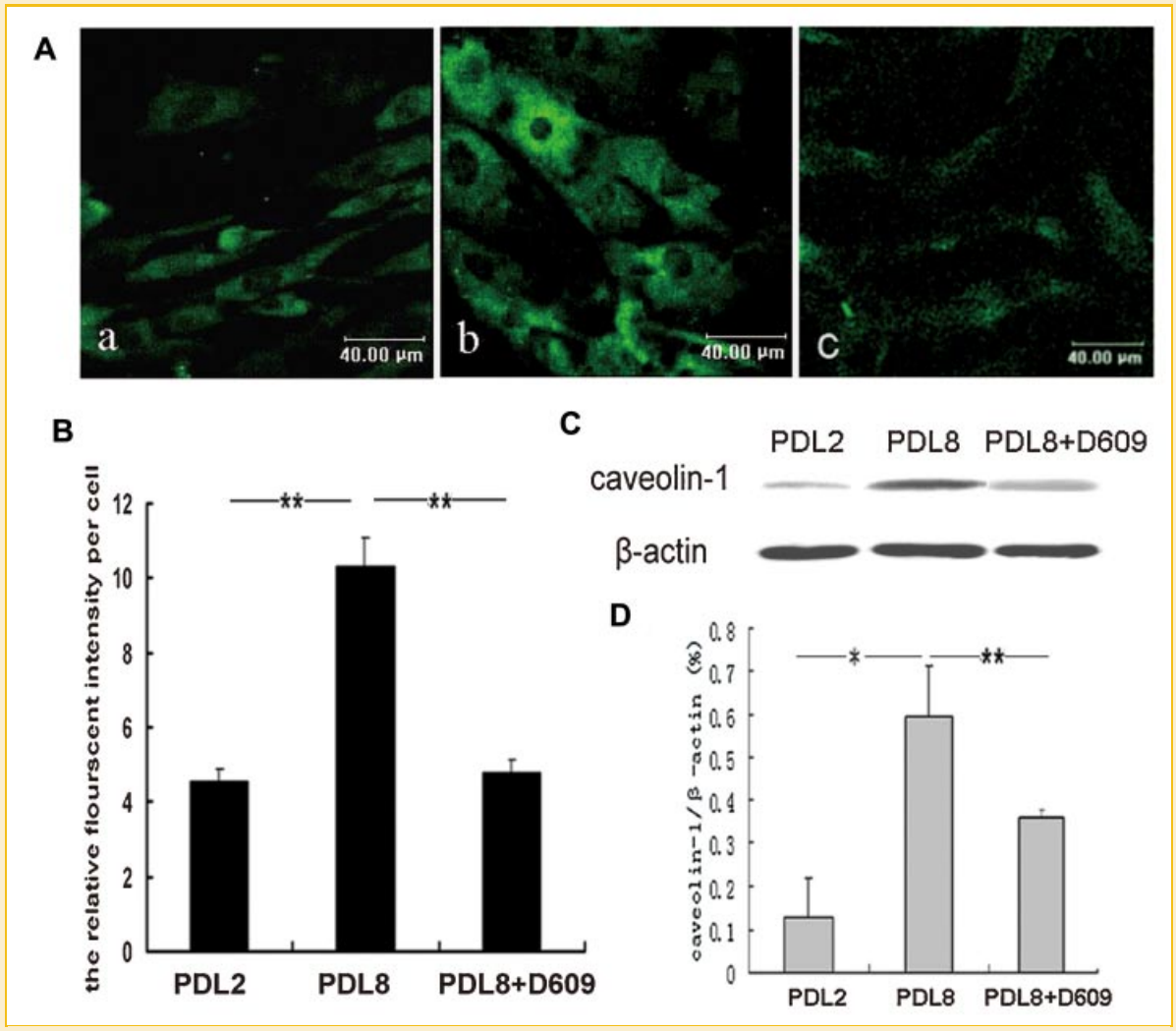

Fig. 6. Caveolin-1 level during BMSC senescence. A: Immunocytochemical analysis of relative intensity of caveolin-1 in BMSCs at PDL 2 (a), PDL 8, (b) and (c) PDL 8 with 4 μg/ml D609. B: Quantification of caveolin-1 level ($^{**}P < 0.01$, n = 5). C: Western blot analysis of caveolin-1 protein level. D: Quantification of caveolin-1 protein level. $^{*}P < 0.05$; $^{**}P < 0.01$, n = 4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and ROS in the process. These findings provide new evidence for the molecular mechanism of BMSC senescence to aid in the use of these cells in tissue engineering.

SA-β-Gal staining is used to test the senescence of several kinds of cells because beta-galactosidase activity is associated with cell senescence in vitro at PH 6. Many studies on BMSC senescence have described β-gal activity increased on prolonged BMSC cultivation [Park et al., 2005; Vacanti et al., 2005]. Senescent BMSCs have a diminishing division capacity that can be used as a marker to test cell senescence [Sethe et al., 2006]. In addition, accumulated evidence shows that the chondrogenic, osteogenic and adipogenic potential of BMSCs decrease with senescence [Kretlow et al., 2008]. Therefore, we used these indexes of SA-β-gal staining and differentiation into adipocytes to characterize BMSC senescence.

Telomerase activity is still disputed during BMSC senescence [Sethe et al., 2006], although it is involved in senescence of some cells [Parsch et al., 2004; Torella et al., 2004]. In this study, we examined telomerase activity in PDL 2 and 8 BMSCs. We found no significant alteration in the telomerase activity during BMSC senescence, and D609 did not affect the telomerase activity of PDL 8 BMSCs (data not shown). This finding is consistent with other results of low telomerase activity in BMSCs and no correlation with their senescence [Parsch et al., 2004].

PC-PLC plays a pivotal role in cellular signaling pathways. However, the mammalian PC-PLC has not been cloned, and its sequence is unknown [Liu et al., 2007a]. Thus, the specific PC-PLC inhibitor D609 and activity assay for PC-PLC have been used as tools for investigation of this enzyme in mammalian cells. Previously, we observed decreased PC-PLC activity during senescence of VECs [Liu et al., 2007b]. More interestingly, we found that inhibition of PC-PLC activity by D609 induced BMSC differentiation to neurons in the absence of serum [Wang et al., 2007a, 2008]. In the current study, the activity of PC-PLC was increased during senescence of BMSCs, and D609 suppression of the increased PC-PLC activity inhibited BMSC senescence in the presence of serum. Therefore, PC-PLC could be cell specific during senescence and PC-PLC could

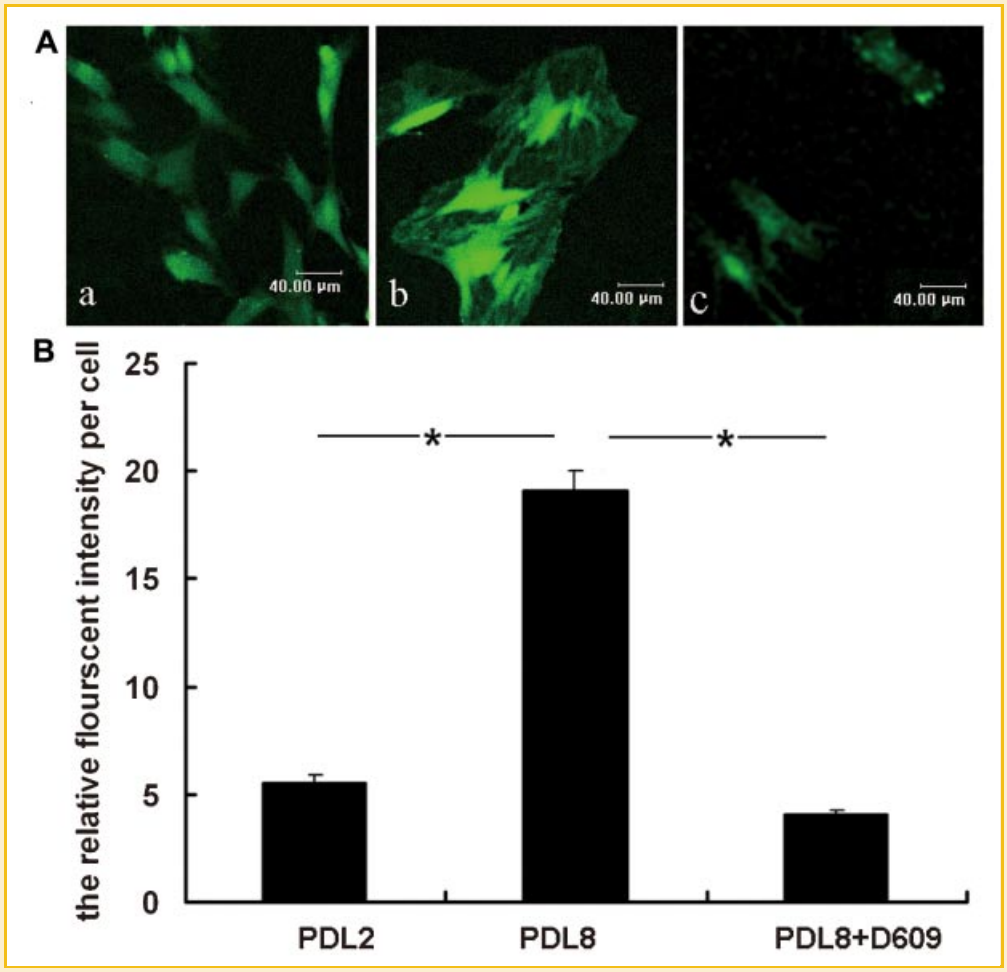

Fig. 7. Intracellular ROS levels in BMSC senescence. A: Fluorescent micrographs of relative intensity of ROS in BMSCs at PDL 2 (a) PDL 8 (b) and (c) PDL 8 with 4 μg/ml D609. B: Quantification of intracellular ROS levels. $^{*}P < 0.05$, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

have distinct functions under different conditions. PC-PLC might be a pivotal factor in the association of senescence and neural differentiation of BMSCs.

Previously, we found the level of integrin β4 increased during the senescence of VECs, which is consistent with our current result. Patched distribution of integrins indicated their active status [Yauch et al., 1997]. The activation can mediate a signal transduction event of broad biological significance, such as in angiogenesis, hemostasis, wound repair, inflammation, and immunity [Liddington and Ginsberg, 2002]. In addition, we discovered that activation of integrin β4 might be involved in VEC apoptosis [Zhao et al., 2005]. In the current study, integrin β4 distributed in clusters in senescent BMSCs, which suggests that the level of integrin β4 was increased and its activity elevated during the BMSC senescence. Importantly, the level of integrin β4 and aggregation decreased after D609 administration, which indicates that PC-PLC mediated BMSC senescence associated with integrin β4.

Caveolae is a special lipid raft in cell membrane. As an important element of caveolae, caveolin can interact with many signal molecules via scaffolding proteins, including G proteins, protein kinase C, Shc, SOS, Raf1 and src-family tyrosine kinases, collected into the caveolae [Brown and London, 1998; Okamoto et al., 1998; Smart et al., 1999; Galbiati et al., 2001]. Upregulated caveolin-1 proteins level was observed in senescent human diploid fibroblasts [Park et al., 2000]. Moreover, loss of adipogenic differentiation potential in senescent human BMSC was found to be mediated by the overexpression of caveolin-1 [Park et al., 2005]. These results are compatible with our; the level of Caveolin-1 in senescent BMSCs was increased and the ability of adipogenic differentiation was correspondingly impaired. More importantly, decreasing the activity of PC-PLC with D609 could inhibit the increased caveolin-1 level in senescent cells. The data suggest that PC-PLC mediated BMSC senescence in association with caveolin-1.

Increased activity of ROS-generating stressors can induce premature senescence [Passos and Von, 2006; Xin et al., 2003]. The accumulation of ROS in senescent cells induces the peroxidation of unsaturated fatty acid, which results in cell damage [Spiteller, 2001]. ROS can limit the proliferation of BMSCs [Meagher et al., 1988]. We also observed a higher level of ROS in senescent BMSCs than in young cells, and inhibition of PC-PLC by D609 could decrease the level of ROS in the senescent cells, which suggests that PC-PLC regulated BMSC senescence in association with ROS.

In summary, during rat BMSC senescence, the activity of PC-PLC and levels of integrin β4, caveolin-1 and ROS are increased, and inhibiting the activity of PC-PLC by D609 can inhibit the senescence and decrease the levels of integrin β4, caveolin-1 and ROS. The data suggest that PC-PLC regulates BMSC senescence through integrin β4, caveolin-1 and ROS.

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