Stromal cell-derived factor 1 protects human periodontal ligament stem cells against hydrogen peroxide-induced apoptosis

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Received April 30, 2016; Accepted March 23, 2017

DOI: 10.3892/mmr.2017.7192

Abstract. Periodontal ligament stem cells (PDLSCs) are considered a promising cell source for dental tissue regeneration. Stromal cell-derived factor 1 [SDF-1, also known as chemokine (C-X-C motif) ligand 12] is regarded as a critical cytokine involved in stem/progenitor cell chemotaxis and homing during tissue regeneration. The present study described a previously unsuspected role for SDF-1 in the protection of PDLSCs against oxidative stress-induced apoptosis. In the present study, apoptosis was induced by exposure of PDLSCs to various concentrations of H_2O_2 for 12 h, following which cell viability was assessed, and cleaved caspase-3 and -9 expression levels were evaluated. To investigate the potential mechanism underlying this protection, the protein expression levels of total and phosphorylated extracellular signal-regulated kinase (ERK), a key protein of the mitogen-activated protein kinase (MAPK) signaling pathway, were examined. The results of the present study revealed that SDF-1 pretreatment increased cell viability following H_2O_2 administration, and downregulated protein expression levels of activated caspase-3 and -9. Furthermore, treatment with SDF-1 increased the phosphorylation of ERK. The protective effect of SDF-1 was partially inhibited by treatment with PD98059, a MAPK/ERK inhibitor, which decreased cell viability. The results of the present study suggested that SDF-1 treatment is a potential strategy to improve the survival of PDLSCs, which may be beneficial for dental tissue regeneration.

Introduction

Dental procedures, including tooth bleaching, laser radiation and exposure to dental materials, elevate levels of intracellular reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2). ROS-induced oxidative stress has been documented to be closely associated with a series of deleterious effects causing degradation of connective tissue and modification to the periodontal structures (1). Therefore, further studies on the effect of oxidative stress on periodontal cells may further the understanding of the underlying mechanisms involved in periodontal apoptosis.

Periodontal ligament (PDL) stem cells (PDLSCs) have been reported to have restorative potential for the regeneration of tissues damaged by periodontal diseases (2). PDLSCs possess self-renewal and multipotent capabilities that regenerate cementum- and PDL-like tissues. As well as therapeutic application in dental tissue regeneration, PDLSCs have potential for the repair and regeneration of a variety of non-dental and non-oral tissues, including bone, cartilage, skeletal muscle, myocardium, spinal cord and peripheral nerves (3).

The chemokine stromal cell-derived factor-1 (SDF-1) is constitutively expressed and produced by PDLSCs. It serves an essential role in stem cell migration and homing by signaling via its cognate receptor, C-X-C chemokine receptor type 4 (CXCR4) (4). There are multiple shared vital functions between SDF-1 and CXCR4, as demonstrated by $CXCR4^{-/-}$ and $SDF\text{-}1^{-/-}$ animals, which exhibit similar phenotypic outcomes and suffer embryonic lethality at similar times during fetal development (5). As well as its role in development, the SDF-1/CXCR4 axis is important for cell survival. It has been demonstrated that SDF-1/CXCR4 signaling stimulates anti-apoptotic pathways in cultured trophoblasts via the mitogen-activated protein kinase (MAPK) signaling pathway (6). A recent study suggested that SDF-1 may regulate monocyte-macrophage differentiation and survival via differential engagement of CXCR4 (7). However, the effects and underlying mechanisms of SDF-1 treatment on survival of PDLSCs under oxidative stress remain largely unknown. The present study demonstrated the positive effects of SDF-1 pretreatment on PDLSC survival following exposure to H_2O_2, through the activation of extracellular signal-regulated kinase (ERK) signaling.

Materials and methods

Isolation of PDLSCs. PDLSCs were isolated and cultured as previously described (8), with slight modifications.

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Key words: periodontal ligament stem cells, stromal cell-derived factor 1, apoptosis, extracellular signal-regulated kinase, hydrogen peroxide

Briefly, permanent teeth were collected from healthy young adults (age, 19-29 years) for orthodontic purposes at the Department of Orthodontics in Second Affiliated Hospital of Zhejiang University (Hangzhou, China). The study protocol was approved by the Ethics Committee of Zhejiang University and written informed consent was obtained from all donors. PDL tissues were scraped from the root surface and cut into fine pieces, followed by digestion for 2 h at 37˚C in Dulbecco's modified Eagle's medium (DMEM)/F-12 (HyClone; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 3 mg/ml collagenase type I (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 4 mg/ml dispase (Gibco; Thermo Fisher Scientific, Inc.). Following filtration through a cell strainer (40-μm pore size; BD Biosciences, Franklin Lakes, NJ, USA), the cells were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (HyClone; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone; Thermo Fisher Scientific, Inc.) and maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assay. Cell viability was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At passage 3, $2x10^4$ PDLSCs were seeded into each well of 96-well plates and incubated for 24 h, following which the medium was replaced with DMEM/F-12 containing 100-500 μM H_2O_2 (Sigma-Aldrich; Merck KGaA) for 12 h. Cell viability was determined by incubating with 5 mg/ml MTT solution at 37˚C for 4 h. Formazan crystals were subsequently dissolved with dimethyl sulfoxide, and the absorbance of each well was measured at a wavelength of 570 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Inc.). All experiments were performed in quintuplicate for each dose. Cells cultured in DMEM/F-12 only served as a control.

The effect of SDF-1 on apoptosis was evaluated by adding recombinant human SDF-1a (PeproTech, Inc., Rocky Hill, NJ, USA) to PDLSCs at a final concentration of 50 ng/ml for 2 h prior to exposure to 100-500 μM H_2O_2 for a further 12 h.

For ERK inhibition experiments, SDF-1-pretreated PDLSCs were treated with 100-500 μM H_2O_2 together with the ERK inhibitor PD98059 (10 μM; Sigma-Aldrich; Merck KGaA) for 12 h, and the MTT assay was subsequently performed. Equivalent dilutions of PBS served as controls.

Knockdown of SDF-1 using small interfering (si)RNA. SDF-1 siRNA (5'-CGTCAAGCATCTCAAAAT-3') or scramble control siRNA were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. PDLSCs ($1x10^5$) were seeded into a 35-mm culture dish. A total of 24 h later, cells were transfected with 160 nM siRNA in Opti-MEM® medium (Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were performed to determine the RNA interference efficacy 48 h after transfection. Also 48 h after transfection, SDF-1 downregulated PDLSCs were treated with 400 μM H_2O_2 for 12 h, and total cellular proteins were extracted for western blot analysis.

Table I. Primary antibodies used in the present study.

Antigen	Cat. no.	Raised in	Dilution	Supplier
SDF-1	ab155090	Rabbit	1:10,000	Abcam
Caspase-3	ab2302	Rabbit	1:200	Abcam
Caspase-9	ab63488	Rabbit	1:1,000	Abcam
ERK	4695	Rabbit	1:2,000	Cell Signaling Technology, Inc.
p-ERK	4370	Rabbit	1:1,000	Cell Signaling Technology, Inc.
GAPDH	ab181602	Rabbit	1:10,000	Abcam

Abcam, Cambridge, UK; Cell Signaling Technology, Inc., Danvers, MA, USA. SDF-1, stromal cell-derived factor 1; ERK, extracellular signal-regulated kinase; p, phosphorylated.

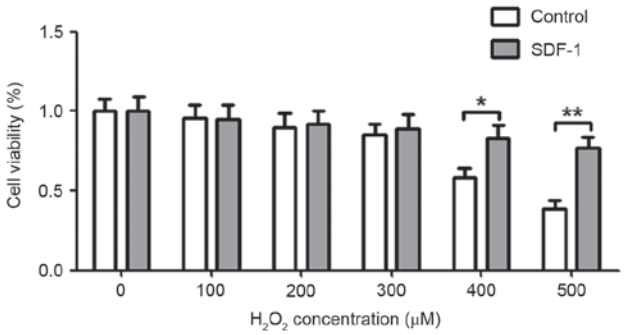

Figure 1. Protective effect of SDF-1 on H_2O_2-induced cytotoxicity in PDLSCs. PDLSCs were cultured for 12 h in presence of 0-500 μM H_2O_2 with or without pretreatment with 50 ng/ml SDF-1 for 2 h. The viability of PDLSCs was measured by an MTT assay. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05; **P<0.01. SDF-1, stromal cell-derived factor 1; PDLSCs, periodontal ligament stem cells.

RNA isolation and RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (2 μg) from each sample was subjected to RT using the PrimeScript™ RT Reagent kit with Moloney Murine Leukemia Virus Reverse Transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). The quantification of human SDF-1 and GAPDH was performed by qPCR on an Applied Biosystems 7500 Real-Time PCR system using a SYBR® Premix Ex Taq kit (Takara Biotechnology Co., Ltd.). PCR reactions were 40 cycles of denaturation at 94˚C for 15 sec, 30 sec annealing and extension at 60˚C. Each value was normalized to β-actin. The relative expression level of the genes was calculated using 2-ΔΔCt method as previously described (9,10). Primer sequences were as follows: Forward, 5'-AGAATTCATGAA CGCCAAGG-3' and reverse, 5'-AGGATCCTCACATCTTGA ACC-3' for SDF-1; forward, 5'-AGGGCTGCTTTTAACTCT GGT-3' and reverse, 5'-CCCCACTTGATTTTGGAGGGA-3' for GAPDH.

Western blot analysis. PDLSCs were seeded into 6-well plates at a density of $5x10^5$ cells/well and cultured for 24 h. H_2O_2 was added at a final concentration of 400 μM for 12 h,

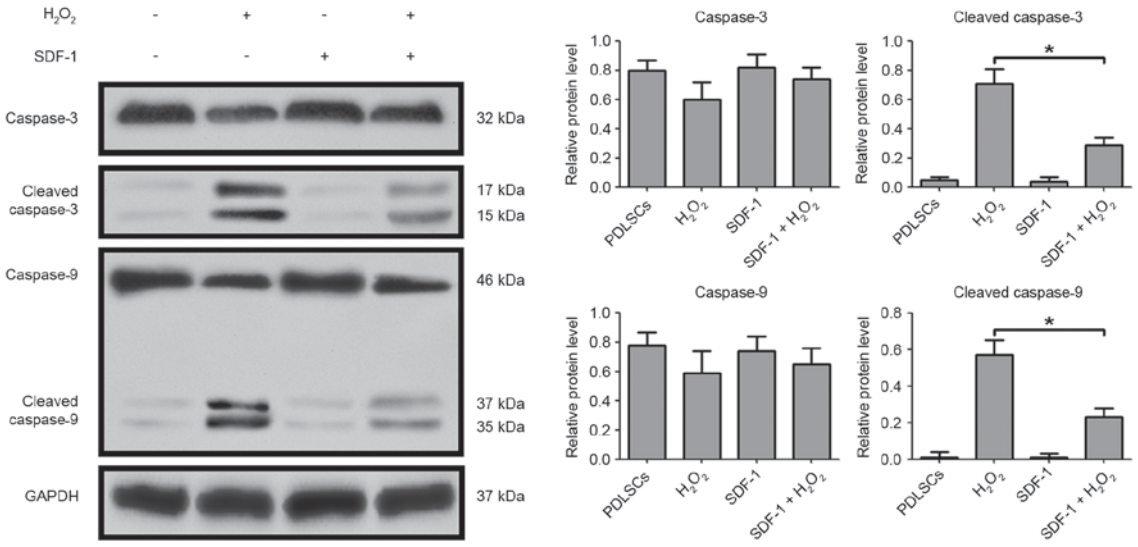

Figure 2. Western blot analysis of caspase-3 and -9 protein expression levels in total cell lysates. Periodontal ligament stem cells were untreated, treated with 400 μM H_2O_2 for 12 h or pretreated with 50 ng/ml SDF-1 for 2 h prior to treatment with 400 μM H_2O_2 for 12 h. GAPDH served as a loading control. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05. SDF-1, stromal cell-derived factor 1.

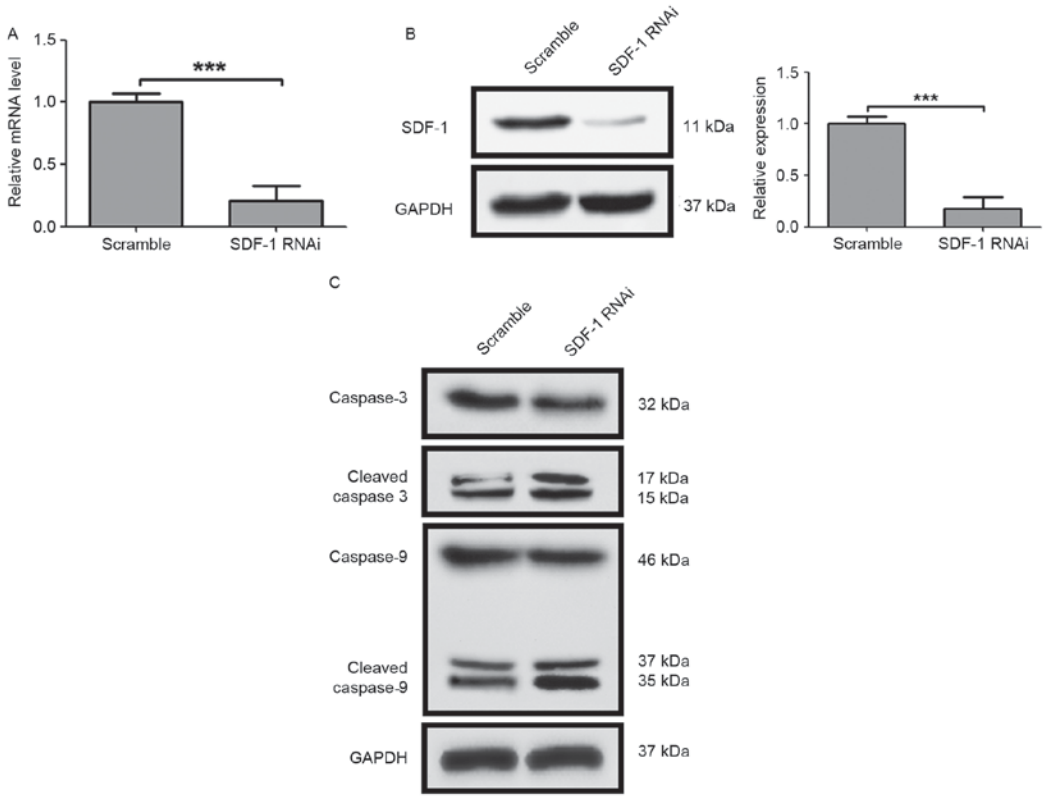

Figure 3. Downregulation of SDF-1 sensitizes PDLSCs to H_2O_2-induced caspase activation. A total of 48 h after transfection with SDF-1 or scramble control siRNA, SDF-1 expression was decreased at the (A) mRNA and (B) protein levels in PDLSCs. GAPDH served as an internal control. (C) SDF-1 knockdown increased the activation level of caspases in PDLSCs. PDLSCs were treated with 400 μM H_2O_2 for 12 h, 2 days following transfection with SDF-1 or scramble control siRNA, and protein expression levels of pro- and cleaved forms of caspase-3 and -9 were determined by western blot analysis. Data are presented as the mean ± standard deviation of three independent experiments. ***P<0.001. SDF-1, stromal cell-derived factor 1; PDLSCs, periodontal ligament stem cells; siRNA, small interfering RNA.

following which cells were lysed and protein concentration was determined using a Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Total proteins (40 μg per lane) were loaded onto 12% SDS-PAGE gels, separated by electrophoresis and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk in 1XTris Buffered Saline Tween-20 and probed with the primary antibodies listed in Table I for

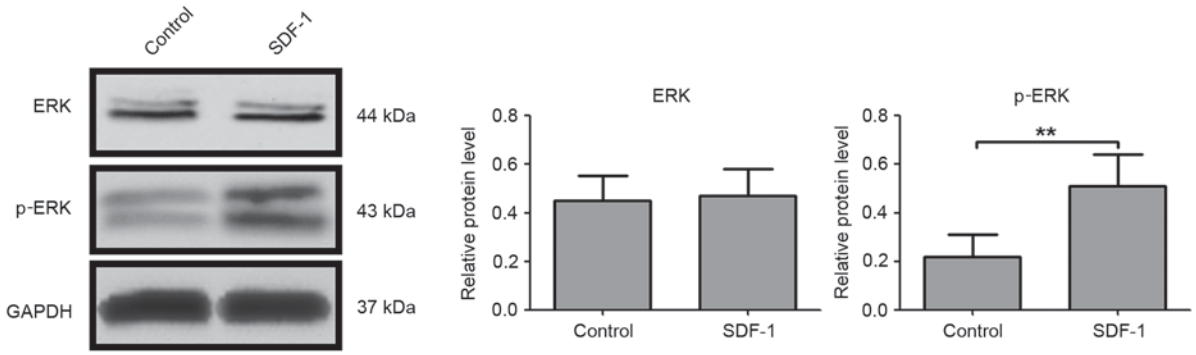

Figure 4. SDF-1 regulates the activation of the ERK signaling pathway. Western blot analysis of ERK and p-ERK proteins in periodontal ligament stem cells treated with 50 ng/ml SDF-1 for 2 h. GAPDH served as a loading control. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01. SDF-1, stromal cell-derived factor 1; ERK, extracellular signal-regulated kinase; p, phosphorylated.

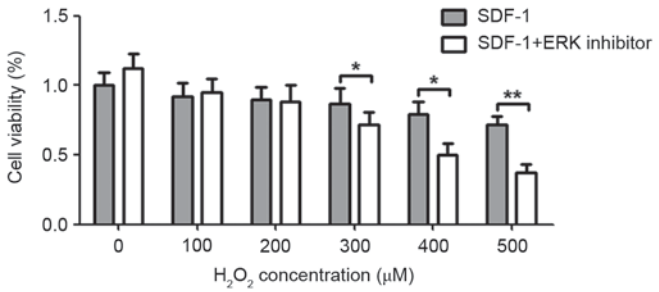

Figure 5. Inhibition of the ERK signaling pathway suppresses the increased survival in SDF-1-pretreated cells under oxidative stress. PDLSCs were pretreated with 50 ng/ml SDF-1 for 2 h prior to culture with 0-500 μM H_2O_2 for a further 12 h. To block ERK signaling, SDF-1 pretreated cells were cultured with 0-500 μM H_2O_2 and 10 μM ERK inhibitor PD98059 for 12 h. The viability of PDLSCs was measured using an MTT assay. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05; **P<0.01. ERK, extracellular signal-regulated kinase; SDF-1, stromal cell-derived factor 1; PDLSCs, periodontal ligament stem cells.

overnight at 4˚C, followed by a horseradish peroxidase-conjugated secondary antibody (65-6120; 1:20,000; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. The protein bands were detected by Pierce™ Western Blot Signal Enhancer (Thermo Fisher Scientific, Inc.), and densitometry was performed using Image J software version 1.50b (National Institutes of Health, Bethesda, MD, USA). Experiments were repeated at least three times.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was evaluated using two-tailed Student's t-test or one-way analysis of variance. Multiple comparisons between the groups was performed using the S-N-K method. P<0.05 was considered to indicate a statistically significant difference.

Results

SDF-1 inhibits H_2O_2-induced cytotoxicity. To evaluate H_2O_2-induced cytotoxicity, PDLSCs were treated with 0, 100, 200, 300, 400 or 500 μM H_2O_2 for 12 h and the cell viability was determined by MTT assay. H_2O_2 treatment reduced cell viability in a dose-dependent manner (Fig. 1). Following treatment with 400 μM H_2O_2 57.4±5.9% of cells survived; this concentration was therefore used for subsequent experiments. The viability of the SDF-1 pretreated cells was similar to untreated control cells and H_2O_2-treated cells when exposed to H_2O_2 concentrations ≤300 μM. However, cell death induced by 400 and 500 μM H_2O_2 was significantly ameliorated by SDF-1 pretreatment.

SDF-1 pretreatment decreases caspase activity. Activation of the caspase cascade is the most important component of programmed cell death. To investigate whether the protective effects of SDF-1 are associated with altered caspase activation, pro- and cleaved caspases were detected by western blot analysis in PDLSCs cultured with 400 μM H_2O_2 for 12 h with or without SDF-1 pretreatment. The protein expression levels of cleaved caspase-3 and -9 significantly increased following treatment with H2O2 (Fig. 2). Pretreatment with 50 ng/ml SDF-1 attenuated this increase, but did not alter the protein expression levels of pro-caspase-3 and -9 (Fig. 2). These data indicated that the protective effect of SDF-1 may be at least partially due to the suppression of caspase activation.

Depletion of SDF-1 sensitizes PDLSCs to H_2O_2-induced apoptosis. To further confirm the role of SDF-1 in H_2O_2-induced apoptosis, SDF-1 or scramble control siRNA was transfected into PDLSCs. After 2 days, mRNA and protein expression levels of SDF-1 in the transfected PDLSCs were measured. Following silencing, the mRNA (Fig. 3A) and protein (Fig. 3B) expression levels of SDF-1 were reduced by 89±12 and 92±11%, respectively, compared with scramble control. Western blot analysis revealed that, compared to scramble control cells, SDF-1 knockdown cells had greater protein expression levels of activated caspase-3 and -9 following H_2O_2 treatment (Fig. 3C). These results suggested a specific contribution of SDF-1 in protecting PDLSCs from H_2O_2-induced apoptosis.

SDF-1 induces the activation of the ERK signaling pathway. The signaling pathway responsible for SDF-1 protection against apoptosis was subsequently investigated. As activation of the ERK signaling pathway has been demonstrated to inhibit apoptosis in response to a wide range of stimuli, including oxidative stress (11), it was hypothesized that ERK may be

involved in SDF-1-mediated protection against H_2O_2-induced cytotoxicity. To test this hypothesis, protein expression levels of the activated and inactivated forms of ERK were examined in PDLSCs treated with 50 ng/ml SDF-1 for 2 h (Fig. 4). Densitometry analysis revealed that SDF-1 treatment activates ERK via an increase in the protein expression levels of phosphorylated ERK.

SDF-1-mediated protection against H_2O_2-induced cytotoxicity of PDLSCs is suppressed by inhibition of ERK. To further investigate whether the ERK signaling pathway is critical for the anti-apoptotic effect of SDF-1 on PDLSCs, the effect of PD98059, an ERK-specific inhibitor, on SDF-1-induced cell survival was assessed. Addition of 10 μM PD98059 to PDLSCs significantly inhibited the protective effect of SDF-1 pretreatment against H_2O_2 concentrations ≥300 μM (Fig. 5). These results supported the role of the ERK signaling pathway in the increased survival mediated by SDF-1 pretreatment. Comparisons between normal PDLSCs and SDF-1 pretreatment after ERK inhibition were not performed as the major focus of the present study was to validate the involvement of ERK pathway in SDF-1-mediated cytoprotection.

Discussion

The SDF-1/CXCR4 axis has been reported to be associated with multiple biological processes, including cell survival, proliferation, adhesion and growth factor secretion (12). SDF-1 is vital in myocardiocytes (13), myeloid cells, neurons (14), leukocytes (15) and muscle precursors (16), and is particularly associated with the promotion of cell survival. The findings of the present study indicated that SDF-1 pretreatment may improve the efficacy of PDLSC-associated tissue regeneration.

Recently, PDLSCs have been investigated for their potential application in regenerative medicine, due to their availability and multipotency (17). Various physiological microenvironments, including mechanical stress and hypoxia, serve a vital role in PDLSC-associated dental tissue regeneration (18). As cell survival is necessary for dental tissue regeneration by PDLSCs, numerous strategies have been investigated to improve the resistance of stem cells to lethal stimuli. Upregulation of pro-survival molecules, including protein kinase B, Pim-1, B-cell lymphoma 2 (Bcl-2) and stem cell factor, protect against apoptosis in stem cells (19-21). In addition, pharmacological pretreatment with growth factors or cytokines may improve the regenerative capability of stem cells (22).

The present study demonstrated that SDF-1 pretreatment increased PDLSC viability in response to oxidative stress, consistent with recent findings in mesenchymal stem cells (MSCs) (23). It was also reported that overexpression of SDF-1 in MSCs significantly increased cell survival after transplantation into rats with acute myocardial infarction (24), indicating a beneficial role of SDF-1 in stem cell-based therapy. In addition, the present study revealed that exposure to H_2O_2 led to an increase in the protein expression levels of cleaved caspase-3 and -9, which were decreased by SDF-1 pretreatment. Increased levels of activated caspases were additionally detected when SDF-1 was silenced in PDLSCs, which provided further evidence supporting the protective role of SDF-1.

There is evidence that the cytoprotective effects of SDF-1 may be conferred via the activation of intracellular pro-survival kinases (25). Although the specific signaling mechanism remains unclear, it is hypothesized to involve the MAPK signaling pathway and exert protection by deactivating Bcl-2 family pro-apoptotic proteins (26). The present study demonstrated that SDF-1 pretreatment activated ERK. Using a specific inhibitor of ERK, the contribution of this kinase to the survival of H_2O_2-treated PDLSCs was analyzed. Inhibition of ERK significantly decreased the survival of SDF-1 pretreated cells in response to oxidative stress. This finding supported and extended the hypothesis of Xia *et al* (27), which stated that the dynamic balance between ERK and its activated form is important in determining cell survival or apoptosis.

In conclusion, the results of the present study suggested that exogenous SDF-1 exerts effective protection against H_2O_2-induced apoptosis. This protection, in primary human PDLSCs, occurs via the activation of the ERK signaling pathway. Thus, SDF-1 could be a novel regime for improving PDLSCs-based cellular therapy.

Acknowledgements

The present study was supported by the Administration of Traditional Chinese Medicine of Zhejiang Province (grant no. 2014ZA069) and the Department of Education of Zhejiang Province (grant no. Y201534720).

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