Differential effects of TGF-β1 and FGF-2 on SDF-1α expression in human periodontal ligament cells derived from deciduous teeth in vitro

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Received December 29, 2011; Accepted February 2, 2012

DOI: 10.3892/ijmm.2012.957

Abstract. Stromal cell-derived factor (SDF)-1α has been reported to play a crucial role in stem cell homing and recruitment to injured sites. However, no information is available about its role in periodontal tissues. The aim of this in vitro study was to investigate the effects of basic fibroblast growth factor (FGF-2) and transforming growth factor (TGF)-β1 on SDF-1α expression in immortalized periodontal ligament (PDL) cells derived from deciduous teeth (SH9 cells). Real-time PCR and western blot analyses showed that SDF-1α mRNA expression in SH9 cells was markedly inhibited by FGF-2 treatment for 48 h. SU5402, which directly interacts with the catalytic domain of the FGF receptor 1 (FGFR1) and suppresses its phosphorylation, inhibited the FGF-2-related decrease in SDF-1α expression. These results suggest that FGF-2 signaling via the FGFR1 pathway inhibits SDF-1α expression. Conversely, SDF-1α expression in SH9 cells was increased by TGF-β1 treatment for 12 h. Western blot analysis showed that this treatment induced Smad2/3 phosphorylation. A time-course experiment showed that SDF-1α expression levels reached a maximum 12 h after the TGF-β1 treatment and returned to basal levels by 48 h. Real-time PCR analysis showed that Smad7 mRNA expression peaked by 6 h after TGF-β1 treatment. Since Smad7 siRNA downregulated Smad7 expression by approximately 2.5-fold compared with the negative control siRNA, the induction of SDF-1α expression was prolonged. Furthermore, treatment of SH9 cells with TGF-β1 for 12 h induced transwell migration of UE7T-13 cells, which are mesenchymal stem cells derived from human bone marrow. Therefore, SDF-1α may play an important role in stem and progenitor cell recruitment and homing to injured sites in the periodontal ligament, and regulation of SDF-1α expression may be a useful tool in cell-based therapy for periodontal tissue regeneration.

Introduction

The periodontal ligament (PDL) is located between the tooth root and the alveolar bone (1). It comprises a heterogeneous cell population that includes fibroblasts, cementoblasts, osteoblasts, endothelial cells, epithelial cell rests of Malassez, osteoclasts, and progenitor or stem cells (2). The PDL has an important role not only in supporting the tooth but also in contributing to tooth nutrition, homeostasis, and tissue repair (3,4). In vitro studies have demonstrated that PDL cells can differentiate into cementoblastic or adipogenic cells (5). Therefore, the PDL probably contains pluripotent progenitor cells or putative stem cells. These cells can migrate to sites of injury, differentiate, and regenerate periodontal tissues. However, the detailed mechanism of stem and progenitor cell migration in the PDL has yet to be fully understood.

Stromal cell-derived factor (SDF)-1α (or CXCL12) is an α-chemokine that strongly chemotactically attracts mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) through interaction with its unique receptor, CXCR4 (6). This ligandreceptor pair plays a crucial role in the mobilization, migration, homing, proliferation, and differentiation of MSCs and EPCs (6-9). In adults, tissue repair and regeneration following injury are thought to involve the selective recruitment of circulating or resident stem cell populations. The importance of SDF-1α in stem and progenitor cell recruitment is evident by the fact that its expression in injured tissue correlates with the recruitment of adult stem cells and tissue regeneration (6-9). Therefore, while the role of SDF-1α in coordinating tissue repair has been established, its role in the PDL remains to be elucidated.
At present, bioactive soluble factors such as cytokines and growth factors are being evaluated for clinical use in the regeneration of periodontal tissue damaged or lost due to periodontitis. Of these factors, both the basic fibroblast growth factor (FGF-2) and the transforming growth factor (TGF)-β1 are multifunctional growth factors that have various effects, including the induction of proliferation and differentiation of a wide range of mesodermal and neuroectodermal cells (10,11). However, their effects on SDF-1α expression in PDL cells are currently unknown.

SH9 cells are immortalized human PDL cells derived from deciduous teeth by the induction of the human telomerase reverse transcriptase (hTERT) gene (12). In this in vitro study, we used SH9 cells to investigate the effects of FGF-2 and TGF-β1 on SDF-1α expression in PDL cells.

Materials and methods

Cell culture. SH9 human PDL cells were maintained in α-modified minimum essential medium (α-MEM; Gibco, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 50 μg/ml streptomycin, and 50 U/ml penicillin. UE7T-13 cells, which are human bone marrow-derived MSCs infected with retroviruses expressing papillomavirus E7 and hTERT to prolong their lifespan (13,14), were purchased from the Health Science Research Resources Bank (JCRB1154, Japan Health Sciences Foundation, Tokyo, Japan) and cultured in α-MEM. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Reverse transcription polymerase chain reaction (RT-PCR). After treatment with 10 ng/ml FGF-2 or 25 ng/ml TGF-β1 for the indicated times, total RNA of SH9 cells was isolated using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed to first-strand cDNA using a PrimeScript RT reagent kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocols. Subsequently, cDNA samples were amplified with specific primer pairs for SDF-1α and β-actin (Table I). PCR was performed for 33 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 1 min. Then 8 μl of the PCR product was mixed with 2 μl of bromophenol blue-loaded buffer [0.25% bromophenol blue, 0.35% xylene cyanol, 40% (wt/vol) sucrose in water] and electrophoretically separated in a 1% (wt/vol) agarose gel in TAE buffer. After staining with 0.5 μg/ml ethidium bromide, bands of the PCR products were visualized with ultraviolet light and images were captured on polaroid film.

Western blotting. Following treatment with 10 ng/ml FGF-2 for 7 days, the conditioned medium from the SH9 cell culture was collected to obtain SDF-1α samples. For Smad2/3 samples, SH9 cells were lysed with RIPA buffer after treatment with TGF-β1. Then, 20 μl of the conditioned medium or 30 μg of the cell lysis sample was dissolved in sodium dodecyl sulfate (SDS) buffer without dithiothreitol (DTT), incubated at 95°C for 5 min, electrophoresed on 10% SDS-polyacrylamide gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After being blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated with mouse anti-human SDF-1α antibody (R&D Systems, Abingdon, UK) and subsequently with anti-mouse secondary antibodies (Zymed Laboratories, San Francisco, CA, USA). Specific protein bands were detected by using an enhanced AP conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (15,16).

Real-time PCR. A Thermal Cycler Dice real-time system (Takara Bio) was used for real-time PCR. The cDNAs were amplified with SYBR® Premix Ex Taq and specific primer pairs for SDF-1α, Smad7, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Table I). The PCR conditions were: 10 sec at 95°C, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, and finally, 15 sec at 95°C and 30 sec at 60°C.

RNA interference. Transfection of short interfering RNAs (siRNAs) was performed according to the Lipofectamine RNAiMAX protocol (Invitrogen). In brief, SH9 cells were transiently transfected with siRNA against Smad7 (Stealth RNAi, Invitrogen). Forty-eight hours after transfection, the cells were treated with 25 ng/ml TGF-β1 for 0, 3, 6, 12, 24, or 48 h. Then, real-time PCR was performed as described above.

Transwell migration assay. SH9 cells were plated at a density of 2.0×10⁴ cells/cm² in 24-well tissue culture plates. When 12 h later the cells reached confluence, they were treated with 50 ng/ml TGF-β1 for 12 h. Then, the wells were washed thrice with phosphate-buffered saline (PBS), and the medium was replaced with α-MEM containing 2% FBS. UE7T-13 cells were used for the assay. First, 2.0×10⁴ UE7T-13 cells were plated on the upper wells of 6.5-mm-diameter cell culture inserts (BD Biosciences, Bedford, MA, USA) with 8-μm-pore filters. Following incubation for 6 h at 37°C, the cells that had not migrated were scraped with a cotton swab, and the filters were stained with a Diff-Quik kit (Sysmex, Hyogo, Japan). The number of cells that had migrated to the lower side of the filters was counted under a light microscope (x400 magnification).

Statistical analysis. Data are expressed as the means ± (SEM) of triplicate experiments and were analyzed by the Student's t-test. P<0.01 indicates a statistically significant difference.
Results

SDF-1α expression in SH9 cells is inhibited by FGF-2 treatment. SH9 cells were treated with 10 ng/ml FGF-2 and/or 10 µM SU5402 for 48 h, and their morphology was examined. The cells acquired a spindle cell-type morphology after the FGF-2 treatment (Fig. 1C) although the morphology was not different from that of the control cells (Fig. 1A). SDF-1α mRNA expression decreased after the FGF-2 treatment (Fig. 1E), and the decrease occurred in a time-dependent manner (Fig. 1F).

SDF-1α production in the SH9 cell culture is reduced by FGF-2 treatment. SDF-1α protein in the conditioned medium from the SH9 cell culture was detected by western blotting. Treatment with FGF-2 led to a decrease in SDF-1α production compared with the control condition (Fig. 2A and B), similarly to the significantly decreased mRNA expression (Fig. 1E and F).

TGF-β1 induces SDF-1α expression in SH9 cells via the Smad2/3 pathway. Real-time PCR analysis revealed that TGF-β1 treatment for 12 h induced SDF-1α expression in SH9 cells (Fig. 3A). SDF-1α expression did not change following treatment with 10 µM SB431542 alone or with both TGF-β1 and SB431542 (Fig. 3A). To confirm this effect of TGF-β1, the levels of Smad2/3 and phosphorylated Smad2/3 were evaluated by western blot analysis. Although the total level
Figure 3. Effects of TGF-β1 on SDF-1α and Smad2/3 in SH9 cells. (A) Real-time PCR analysis showed that treatment with 25 ng/ml TGF-β1 for 12 h increased SDF-1α expression in SH9 cells; however, in the presence of 10 µM of SB431542 and both TGF-β1 and SB431542, SDF-1α expression was unaffected. (B-D) Smad2/3 and phosphorylated Smad2 (p-Smad2) expressions were normalized to that of GAPDH and are shown as a percentage of the control (0 h). Data are the means ± (SEM) of triplicate experiments; *P<0.01 indicates a statistically significant difference.

Figure 4. Effects of TGF-β1 on (A) SDF-1α and (B) Smad7 expressions in SH9 cells. SH9 cells were treated with 25 ng/ml TGF-β1 for 3, 6, 12, 24, or 48 h. SDF-1α and Smad7 expressions were normalized to that of GAPDH and are shown as a percentage of the control (0 h). (C) Transfection of siRNA against Smad7 prolonged the TGF-β1-induced increase in SDF-1α expression in SH9 cells; the negative control did not have any effect on the prolonged expression of SDF-1α. (D) Transfection of siRNA against Smad7 suppressed the induction of Smad7 expression. Data are the means ± (SEM) of triplicate experiments; *P<0.01 indicates a statistically significant difference.
UE7T-13 cell migration is induced in the SH9 cell culture treated with TGF-β1. Migration of UE7T-13 cells was induced when culture inserts with the cells were placed into wells containing SH9 cells treated with 50 ng/ml TGF-β1 for 12 h (Fig. 5).

Discussion

To date, information regarding the expression and regulation of SDF-1α in periodontal tissues is lacking. To the best of our knowledge, this is the first study to show that the expression of SDF-1α in PDL cells is regulated by FGF-2 and TGF-β1. FGF-2 inhibited SDF-1α expression to approximately 10% of the control level by 48 h, and TGF-β1 increased it to approximately 6-fold of the control level between 6 and 12 h after treatment. These results suggest that SDF-1α may play an important role in stem and progenitor cell recruitment and homing to injured sites in the PDL.

SDF-1α is a small (8-13 kDa) and multifunctional cytokine that is constitutively expressed and secreted by several tissues, including the endothelium and stromal cells (17). Expression of SDF-1α is upregulated to attract EPCs and MSCs in response to damage of various tissues or organs (8). In the present study, SDF-1α was constitutively expressed in PDL cells (Fig. 2A) and its expression was modulated by the treatments with FGF-2 and TGF-β1. PDL cells may possess regulatory mechanisms to induce and/or suppress various growth factors, including FGF-2 and TGF-β1 (18,19). Since both FGF-2 and TGF-β1 are expressed in periodontal tissues by occlusal stimuli and orthodontic force, respectively (20,21), the PDL may maintain itself via the expression of cytokines, such as SDF-1α, FGF-2 and TGF-β1.

FGF-2 affects the functional ability of pluripotent progenitor cells or putative stem cells in the PDL. It facilitates the differentiation of these cells to endothelial-like cells (15,16). In the present study, FGF-2 inhibited SDF-1α expression in SH9 cells. As SU5402 is an FGF receptor (FGFR)-specific tyrosine kinase inhibitor (22), it suppressed the FGF-2-induced morphological changes, and decreased SDF-1α expression, in SH9 cells (Fig. 1). Therefore, FGF-2 affected SDF-1α expression via the FGFR pathway. However, whether or not intracellular signaling depends on the inhibition of SDF-1α expression in SH9 cells remains unclear.

TGF-β1 is a member of the TGF-β superfamily, which mediates multiple biological processes including bone formation (23). TGF-β1 plays a pivotal role in the commitment and differentiation of various cell lineages (23). SB431542 is a small molecule that acts as a specific inhibitor of TGF-β type I receptor activin receptor-like kinase 5 (ALK5) (23). Considering that treatment with both TGF-β1 and SB431542 suppressed the TGF-β1-induced increase in SDF-1α expression in SH9 cells, it appears that TGF-β1 affects SDF-1α expression via ALK5. Moreover, TGF-β1 affects Smad2/3 phosphorylation (Fig. 3). Transient transfection of siRNA against Smad7 prolonged the TGF-β1-induced increase in SDF-1α expression in SH9 cells. These findings showed that the induction of SDF-1α expression in SH9 cells by treatment with TGF-β1 is regulated via the ALK5-Smad2/3 pathway (24). Finally, SH9 cells treated with TGF-β1 for 12 h could induce the migration of MSCs (Fig. 5).

Figure 5. Results of the transwell migration assay show that SH9 cells induce mesenchymal stem cell (MSC) migration. (A) After SH9 cells in 24-well plates were treated with 50 ng/ml of TGF-β1 for 12 h (step 1), the culture medium in the wells was removed. The cells were washed thrice with PBS, and transwell cell culture inserts were placed into the wells (step 2). UE7T-13 cells were plated onto the upper side of the inserts (step 3) and cultured for 6 h (step 4). Finally, the cells on the lower side of the inserts were counted (B). Data are the means ± (SEM) of triplicate experiments; *P<0.01 indicates a statistically significant difference.

of Smad2/3 remained the same, the level of phosphorylated Smad2/3 increased and peaked 1 h after the addition of TGF-β1 (Fig. 3B-D). It then decreased 2 h after the stimulation (Fig. 3D).

**TGF-β1 induced SDF-1α and Smad7 expression in SH9 cells.** As shown in Fig. 4A, SDF-1α expression increased until 12 h and then decreased 48 h after the treatment with TGF-β1. Smad7 expression in SH9 cells also increased by ~5-fold until 6 h after the treatment, and then decreased by 48 h (Fig. 4B).

**Transfection of Smad7 siRNA prolongs the induction of SDF-1α expression in SH9 cells.** Transfection of siRNA against Smad7 resulted in prolonged induction of SDF-1α expression (~5-fold), even after 48 h following the TGF-β1 treatment (Fig. 4C), but it suppressed Smad7 expression after 3 h (Fig. 4D).
PDL cells are thought to be responsible for homeostasis and regeneration of periodontal tissues (3-5) following injury or inflammation and mechanical stress by orthodontic treatment. Previous studies have indicated that the PDL contains MSCs (5). Bone marrow-derived MSCs are considered a good source of cells for cell-based therapy (25). However, bone marrow aspiration for MSC extraction is an invasive and painful procedure. In addition, the abundance of these cells as well as their proliferation and differentiation potential decline with increasing age (26). Comparatively, MSC isolation from the PDL of deciduous teeth is noninvasive and economical. These cells can be frozen until required in the clinic. Although MSC isolation from the PDL yields only a small number of cells, these cells can be used in cell-based therapy for tissue regeneration as a source of SDF-1α.

In conclusion, SDF-1α expression in PDL cells can be regulated by FGF-2 and TGF-β1, and PDL cells treated with TGF-β1 can induce the migration of MSCs. These results suggest that periodontal tissue can be maintained by SDF-1α expression, and PDL cells isolated from deciduous teeth are beneficial tools for cell-based therapy.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research (nos. 18592026 and 23592896 to A.I., no. 19791370 to N.C., no. 21390548 to M.M., and nos. 18592239 and 22592296 to T.H.); by grants from the Open Research Project (2009-2010), the High-Tech Research Project (2008), and the Medical Innovation by Advanced Science and Technology (MIAST); and a Grant-in-Aid for Strategic Medical Science Research (nos. 18592026 and 23592896 to A.I., no. 19791370 to T.H.); by grants from the Open Research Project (2009-2010), the High-Tech Research Project (2008), and the Medical Innovation by Advanced Science and Technology (MIAST); and a Grant-in-Aid for Strategic Medical Science Research (nos. 18592026 and 23592896 to A.I., no. 19791370 to T.H.); by grants from the Keiryokai Research Foundation (no. 100 to N.C., 2008; no. 103 to A.I.; no. 106 to T.H., 2009).

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