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Vitamin C Treatment Promotes Mesenchymal Stem Cell Sheet Formation and Tissue Regeneration by Elevating Telomerase Activity

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Abstract

Cell sheet engineering has been developed as an alternative approach to improve mesenchymal stem cell-mediated tissue regeneration. In this study, we found that vitamin C (Vc) was capable of inducing telomerase activity in periodontal ligament stem cells (PDLSCs), leading to the up-regulated expression of extracellular matrix type I collagen, fibronectin, and integrin $\beta 1$, stem cell markers Oct4, Sox2, and Nanog as well as osteogenic markers RUNX2, ALP, OCN. Under Vc treatment, PDLSCs can form cell sheet structures because of increased cell matrix production. Interestingly, PDLSC sheets demonstrated a significant improvement in tissue regeneration compared with untreated control dissociated PDLSCs and offered an effective treatment for periodontal defects in a swine model. In addition, bone marrow mesenchymal stem cell sheets and umbilical cord mesenchymal stem cell sheets were also well constructed using this method. The development of Vc-mediated mesenchymal stem cell sheets may provide an easy and practical approach for cell-based tissue regeneration.

With rapid progress in stem cell research, stem cell therapies have become promising therapeutic approaches in clinics. Cell-based regenerative medicine has recently been extensively investigated (Dezawa et al., 2005; Moreau and Xu, 2009; Sonoyama et al., 2006; Liu et al., 2008). However, in past decades, tissue fabrication techniques in regenerative medicine largely relied on scaffold-based approaches (Langer and Vacanti, 1993). Numerous challenges existed in fabricating functional tissue-engineered organs. These barriers include insufficient cell migration into and retention within scaffolds, host inflammatory reactions, limited capabilities to generate microscale vascularization for mass transport, different rates of cell proliferation compared with scaffold degradation, and the inability to generate functional tissues with the architectural complexity of native tissues because of scaffold-based methods. It has been speculated that the use of continuous cell sheets, with the preservation of cellular junctions, endogenous extracellular matrix (ECM),

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and mimicking cellular microenvironments in terms of various mechanical, chemical, and biological properties, may be beneficial for cell transplantation. Okano's group developed a temperature-responsive culture dish that could be used to harvest cultured cells non-invasively as intact sheets, together with their deposited ECM (Okano et al., 1993, 1995; Yang et al., 2007). Cell sheet engineering has been developed as an alternative approach to tissue engineering in corneal, myocardial, hepatic, and periodontal tissues (Nishida et al., 2004; Shimizu et al., 2006; Ohashi et al., 2007; Ding et al., 2010). In particular, corneal reconstruction by cell sheet has been applied in clinical purposes (Nishida et al., 2004). At present, several improvements have been made to harvest the living cell sheet more easily, such as the coating of dishes with a thermo-responsive hydrogel (Chen et al., 2006, 2007) or laminin-5 (Shimizu et al., 2009). However, the entire grafting process remains relatively complicated, time-consuming, and requires special materials. In the latest study, dexamethasone and ascorbic acid phosphate (vitamin C, Vc) were used to create cell sheets to enhance bone formation (Nakamura et al., 2010).

Vc, a common nutrient vital to human health, is a water-soluble vitamin essential for the synthesis and function of immune system factors. Vc also plays a key role in the biosynthesis of collagen and other ECM constituents, and acts as a cofactor in many biological reactions throughout the human body (Stone and Meister, 1962; Nandi et al., 2005; Korkmaz and Kolankaya, 2009). When supplied to the culture medium, Vc can act as a growth promoter to increase cell proliferation and DNA synthesis (Choi et al., 2008). Moreover, the addition of Vc has been shown to inhibit differentiation and up-regulate pluripotency marker expression of Oct4 and Sox2 (Ji et al., 2010; Potdar and D'Souza, 2010). Vc is necessary to biosynthesize ECM, as well as to mimic the *in vivo* physiological environment of mesenchymal stem cells (MSCs) and regulate their proliferation and differentiation. We predict that Vc alone may induce cell sheet formation, which may benefit cell-based tissue engineering. On the basis of this hypothesis, we developed a simple and inexpensive Vc-mediated procedure to obtain PDLSCs sheet, which represents an alternative approach to cell sheet formation, and may have clinical applications in regenerative medicine.

Materials and methods

Cell culture

All protocols for the handling of human tissue were approved by the Research Ethical Committee of Capital Medical University, China. Informed consent was obtained from all donors. Animal study was reviewed and approved by the Animal Care and Use Committee of Capital Medical University.

Extracted human impacted third molars were selected from 16 volunteers in the School of Stomatology, Capital Medical University. Minipig canines were obtained from 9 minipigs. Human bone marrow was collected from 14 human donors undergoing a spine fusion surgical procedure at Beijing Friendship Hospital, which is affiliated with Capital Medical University. Umbilical cords were obtained from 17 donors from Beijing Friendship Hospital. The isolation of human and minipig periodontal ligament stem cells (hPDLSCs and pPDLSCs), human bone marrow mesenchymal stem cells (hBMMSCs), and human umbilical cord mesenchymal stem cells (hUCMSCs) was performed as previously reported (Sonoyama et al., 2006; Ding et al., 2010; Seo et al., 2004; Martino et al., 2009; Park et al., 2007). hPDLSCs, pPDLSCs, hBMMSCs, and hUCMSCs were cultured in alpha-modified Eagle's medium (α -MEM) (Gibco; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco; Invitrogen Corp., Carlsbad, CA, USA), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen Corp., Carlsbad, CA, USA), and then incubated at 37°C in 5% carbon dioxide. 1.0×10^5 cells were cultured

in 60 mm dishes. Different concentrations of Vc (Sigma-Aldrich Corp., St. Louis, MO, USA) were added to the culture medium for induction. All cells used in this study were at 3–4 passages.

Making mesenchymal stem cell sheets

Characterization of MSCs, including expression profiles of surface molecules and multi-lineage differentiation, was performed as previously reported (Liu et al., 2008; Ding et al., 2010). Next, a dose response experiment was performed to test the optimal dose of Vc (0 µg/mL, 5.0 µg/mL, 10.0 µg/mL, 20.0 µg/mL, 50.0 µg/mL). The response experiment was repeated six times for each dose. 1.0×10^5 hPDLSCs were subcultured in 60 mm dishes. Vc (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the culture medium for the duration of the experiment. The cells became confluent after 2–3 days in culture. Confluent cells were cultured for 7–10 days until the cells at the edge of the dishes wrapped, which implied that cell sheets had formed and could be detached. A cell sheet obtained from a temperature-responsive culture dish (UpCell dish) (CellSeed Inc., Shinjuku-ku, Tokyo, Japan) served as a control. Samples of hPDLSCs sheet were processed for histological examination, transmission electron microscopy (TEM), real-time quantitative polymerase chain reaction (qPCR) examination, and transplantation. Finally, hBMMSCs sheet and hUCMSCs sheet were constructed by adding optimal concentrations of Vc to the culture medium. The resulting samples were harvested, fixed with 4% paraformaldehyde, and assessed histologically.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

PDLSCs were seeded on 96-well plates at a cell density of 2×10^3 cells/well and treated with Vc at concentrations of 0, 5.0, 10.0, 20.0 and 50.0 µg/mL, respectively. At 48 h after Vc treatment, the proliferation/survival of the cells was evaluated using the MTT assay. Briefly, the culture medium was replaced with 5 mg/mL MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS, and the plates were incubated for 4 h at 37°C. The precipitate was extracted with DMSO (Sigma-Aldrich Corp., St. Louis, MO, USA) and the optical density was measured at the wavelength of 490 nm.

TEM observation

Harvested hPDLSCs sheets were fixed using 2.5% glutaraldehyde in 0.1 mg/mL sodium cacodylate buffer (pH 7.2) for 2 h at 4°C. After fixation, the samples were rinsed three times with 0.1 mol/L sodium cacodylate buffer (pH 7.2) for 0.5 h. The samples were post-fixed in 2% osmium tetroxide, washed for 1 h, dehydrated in a graded ethanol series, and embedded in Epon 812 resin according to the manufacturer's instructions. Serial 0.5-µm sections were cut and examined using a light microscope, (BHS-RFK; Olympus, Japan) after being stained with 2% toluidine blue for 5 min. For TEM analysis, 70-nm sections were cut, stained with 2% uranyl acetate for 30 min and 2% lead citrate for 5 min, and observed with a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan).

Measuring telomerase activity

Telomerase activity in hPDLSCs was quantified using a Telo TAGGG telomerase PCR ELISA kit (Roche Ltd., F. Hoffmann-La, USA) according to the manufacturer's protocol, with slight modification. In brief, cell lysate was collected from 20.0 µg/mL Vc-treated hPDLSCs at different time points (0 h, 24 h, 48 h) and 15 µL cell lysate was used for PCR amplification of telomeric repeats with the following parameters: 25°C for 60 min; 94°C for 5 min; 40 cycles (94°C for 30s, 50°C for 30s, 72°C for 30s); extension at 72°C for 5 min; followed by holding at 4°C. Five microliters of each PCR product were denatured and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. One

hundred microliters of each hybridization product were immobilized via biotin-labeled primer to a streptavidin-coated microplate. Finally, the probe was visualized using a peroxidase-conjugated DIG antibody, which metabolized TMB substrate to form a colored reaction product; the relative absorbance was measured using an ELISA reader.

Western blot

Human PDLSCs were treated with Vc at a concentration of 20.0 $\mu\text{g/mL}$ in the presence or absence of 1 μM telomerase inhibitor III (Calbiochem Inc., Merck KGaA, CA, USA). Cells were lysed in RIPA buffer with a protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA), and the protein concentration was measured using a BCA protein assay (Thermo Fisher Scientific Inc., Shanghai, China). Thirty micrograms of protein in each lane were separated on a Tris-Glycine SDS-PAGE gel (Invitrogen Corp., Carlsbad, CA, USA) and transferred onto a PVDF membrane, followed by blocking in 5% BSA for 1 h. Membranes were incubated with primary antibody overnight at 4°C, and then with secondary antibody at room temperature for 1 h. Signals were developed on film by exposing the membrane to chemiluminescence HRP substrate (Thermo Fisher Scientific Inc., Shanghai, China). Antibodies used in this work include: hTert (Abcam plc., Cambridge, UK), integrin $\beta 1$, fibronectin (R&D systems, Minneapolis, Minn., USA), beta actin, and HRP-conjugated secondary antibodies to rabbit and mouse (Santa Cruz Inc., California, USA).

Real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). After DNase treatment, 1 μg of the total RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc., Glen Burnie, MD, USA). Real-time PCR was performed in a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR parameters used were: 95°C for 15 s, followed by 60°C for 60 s. The gene primers used in this study are listed in Table 1; β -actin primers were used to normalize samples. The results were evaluated using the Smart Cycler II software program. All experiments were conducted five times.

Transplantation in nude mice

Twenty-four 5-week-old female nude mice were selected for transplantation (12 animals for each transplantation group). A complete Vc-induced hPDLSCs sheet, a hPDLSCs sheet obtained from an UpCell dish, and dissociated hPDLSCs with the same cell numbers seeded on gelfoam (Pharmacia Canada Inc., Ontario, Canada) were transplanted subcutaneously into the dorsal site of nude mice as previously described (Gronthos et al., 2000). At 4 weeks after transplantation, all animals were sacrificed, and the samples were harvested and fixed with 4% paraformaldehyde and assessed histologically.

Goldner's trichrome staining and Sirius red staining

For Goldner's trichrome staining (following the protocol specified in EMS Catalog #26386), the sections were dewaxed, dyed in Bouin's Fluid solution for 1 h at 56°C, cooled, and washed in running tap water until the yellow color disappeared. Next, the sections were placed in Weigert's hematoxylin for 10 min, washed in running tap water for 10 min, stained in ponceau acid fuchsin for 5 min, and washed in 1% acetic acid. Next, the sections were placed in phosphomolybdic acid-orange G solution until collagen was decolorized, and rinsed in 1% acetic acid for 30 sec. Finally, the sections were stained in light green stock solution for 5 min, and rinsed in 1% acetic acid for 5 min. Sirius red staining was conducted as previously described (Zhang et al., 2006); briefly, the sections were dewaxed, dipped into water, and stained with 1 g/L Picric acid-Sirius red at 37°C for 1 h, and then washed with

water. After being cleared and mounted, collagen types and distribution were observed under a polarized light microscope (dark-field).

Transplantation in miniature swine

Nine 12-month-old inbred miniature swine (weighing 40–50 kg) were obtained from the Institute of Animal Science of the Chinese Agriculture University and used for large animal transplantation in this study. Minipig PDLSCs were isolated from canine and cultured as described previously (Sonoyama et al., 2006; Ding et al., 2010). We generated periodontitis lesions in 9 miniature swine as previously reported (Liu et al., 2008; Ding et al., 2010), for a total of 18 defects. These defects were randomly assigned to three groups, each consisting of 6 defects in 3 miniature swine: the Vc-induced autologous PDLSCs sheet group, in which the defects were treated with flap surgery and transplantation of Vc-induced autologous PDLSCs sheets; the UpCell dish PDLSCs sheet group, in which the defects were treated with flap surgery and transplantation of autologous PDLSCs sheets harvested from an UpCell dish; and the Gelfoam scaffolds + dissociated autologous PDLSCs group, in which the defects were treated with flap surgery and transplantation of dissociated PDLSCs combined with gelfoam. At 12 weeks after transplantation, all animals were sacrificed, and the samples were harvested and fixed with 4% paraformaldehyde and assessed histologically.

Statistical analysis

All data obtained were expressed as the mean with standard deviation and ANOVA was used to analyze differences between groups. *P* values less than 0.05 were considered statistically significant.

Results

Vitamin C-induced human periodontal ligament stem cell sheets

We found that low-dose Vc treatment (0 µg/mL, 5.0 µg/mL, or 10.0 µg/mL) could not induce hPDLSCs to form cell sheets (Fig. 1A–C). Hematoxylin and Eosin (H&E) staining revealed that the fragmented cell sheet extended irregularly in 10.0 µg/mL Vc culture (Fig. 1D). However, when treated with 20 µg/mL and 50 µg/mL Vc for 10–13 days in culture, hPDLSCs easily became confluent and wrapped at the dish edge; the entire cell sheets were detached smoothly using a crooked syringe needle (Fig. 1E, F). The morphology of the whole cell sheet was observed, and it was of similar quality as the cell sheet derived from an UpCell dish (Fig. 1G). H&E staining revealed that the harvested whole PDLSCs sheet was two- or three-layered, and spread uniformly as a two-dimensional tissue structure (Fig. 1H). There was no difference between cell sheets grown with 20 µg/mL and 50 µg/mL Vc in terms of cell sheet structure. The success rate for harvesting cell sheets was 100% when 20 µg/mL Vc was added to the culture medium (10/10), but was only 80% using the UpCell dish (8/10). Taken together, Vc can induce PDLSCs to construct high-quality cell sheets at an optimal Vc concentration of 20 µg/mL.

Vc-induced hPDLSCs sheet preserved intercellular junctions with more extracellular matrix and stemness by activating telomerase

TEM examination revealed that Vc-induced hPDLSCs sheet established and retained tight junctions during the culture period (Fig. 2A, B). A great amount of microfilament was observed in the cytoplasm, and exocytosis vesicles were observed near the plasma membrane, demonstrating the sheet's cell proliferation and differentiation characteristics (Fig. 2C). ECM, including collagen I, was observed between cells (Fig. 2D). These data

indicated that the obtained PDLSCs sheet preserved the intercellular junctions and endogenous ECM, and retained their cellular phenotypes.

Next, we evaluated the effect of different concentrations of Vc on PDLSCs proliferation. The results showed that Vc served as a positive modulators of PDLSCs proliferation. 20.0 $\mu\text{g/mL}$ Vc is the optimal concentration (Fig. 3A). Furthermore we explored the effect of Vc on telomerase activity. We observed that telomerase activity in hPDLSCs gradually increased after treatment with 20.0 $\mu\text{g/mL}$ Vc (Fig. 3B). The level of human telomerase reverse transcriptase (hTERT) protein also gradually increased with the presence of Vc (Fig. 3C). Moreover, real-time PCR results revealed that the mRNA levels of ECM elements, including COL1, integrin $\beta 1$, and fibronectin, were higher in 20 $\mu\text{g/mL}$ Vc-induced hPDLSCs sheet compared with hPDLSCs sheet obtained from an UpCell dish and dissociated hPDLSCs (Fig. 3D). The stem cell markers Oct4, Sox2, and Nanog were also increased in 20 $\mu\text{g/mL}$ Vc-induced PDLSCs sheet compared with PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs, while there was no significant difference between PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs (Fig. 3D). The osteogenic markers including RUNX2, ALP, OCN increased in 20 $\mu\text{g/mL}$ Vc-induced PDLSCs sheet compared with PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs, while there was no significant difference between PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs (Fig. 3D). To test the ability to form mineralized matrix, we used alizarin red S staining to assess the capacity. The results implied that 20 $\mu\text{g/mL}$ Vc could induce calcium nodule formation (Fig. 3E). However, higher expression of COL1, fibronectin, and integrin $\beta 1$, was abrogated by telomerase inhibition (Fig. 3F). These results suggested that Vc was capable of enhancing the proliferation capacity and osteogenic differentiation efficiency of PDLSCs, inducing telomerase activity in PDLSCs, inducing the deposition of more ECM, and therefore greater potential for self-renewal and differentiation.

Vc-mediated hPDLSCs sheet enhanced tissue regeneration in nude mice and miniature swine

To investigate the tissue regeneration properties of Vc-induced MSCs in vivo, 1.0×10^5 hPDLSCs were cultured in 60 mm dishes with 20.0 $\mu\text{g/mL}$ Vc for 10 days to make hPDLSCs sheet. Cell sheets harvested from an UpCell dish and dissociated PDLSCs with the same cell numbers were used as controls. Two types of cell sheets and dissociated PDLSCs seeded on an absorbable gelatin sponge were transplanted subcutaneously into separate nude mice. Four weeks after transplantation, the animals were sacrificed and the grafts were harvested for histological analysis. H&E staining revealed that PDLSCs differentiated into odontoblasts/cementoblast-like cells (arrows) that formed much more regular bone/cementum-like matrix (rectangles) in the PDLSCs sheet transplants (Fig. 4A, B). Limited and irregular bone/cementum-like matrix (rectangles) containing odontoblasts/cementoblast-like cells (arrows) and more gelatin sponge scaffold residue were found in the transplants of dissociated PDLSCs (Fig. 4C). Goldner's trichrome staining revealed that bone/cementum-like matrix is blue (rectangles) (Fig. 4D, E, F). Picrosirius-red staining also revealed that there was much more condensed bone/cementum-like matrix generation in PDLSCs sheet transplants (rectangles) (Fig. 4G, H). The same polarized light view indicated that the condensed tissues were full of collagen type I (in red) and type III (in green) (rectangles) (Fig. 4J, K). There were limited amounts of bone/cementum-like matrix regeneration (rectangles) along the surface of gelfoam carriers in dissociated PDLSCs transplants (Fig. 4I); much less new tissue, including collagen type I (in red) and type III (in green) (rectangles); and plenty of remaining gelfoam carriers (no staining) were found when the samples were examined by polarized light (Fig. 4L). The percentages of bone/cementum-like matrix were significantly higher in the Vc-induced PDLSCs sheet and UpCell dish PDLSCs sheet transplant groups ($44.2\% \pm 5.9\%$ and $35.7\% \pm 4.6\%$, respectively)

than in the dissociated PDLSCs transplant group ($12.5\pm4.0\%$); the percentage of bone/cementum-like matrix was significantly higher in the Vc-induced PDLSCs sheet transplant group than in the UpCell dish PDLSCs sheet transplant group (Fig. 4M), indicating that Vc-induced PDLSCs sheet could regenerate more bone/cementum-like matrix in the mouse model.

After in vitro examination and in vivo transplantation in nude mice, we investigated whether Vc-induced autologous PDLSCs sheet could provide a practical approach for functional periodontal tissue regeneration in a large animal model: miniature swine. We first generated periodontitis lesions in miniature swine and then transplanted autologous minipig cell sheets or disassociated cells with gelfoam for tissue regeneration; the animals were sacrificed at 12 weeks post-transplantation.

Experimental tissues were sectioned in the buccal-lingual direction and stained with H&E to provide a view of the entire section. New bone, cementum, and periodontal ligament were regenerated to normal levels in Vc-induced PDLSCs sheet (Fig. 5A), and regeneration was better in the Vc-induced transplanted sheets than in the dissociated PDLSCs transplants (Fig. 5G). The sulcular epithelium was thin and flat in Vc-induced PDLSCs sheet and UpCell PDLSCs sheet transplants (Fig. 5B, E), but was much thicker in dissociated PDLSC transplants (Fig. 5H). Sharpy's fibers formed in Vc-induced PDLSCs sheet (Fig. 5C), UpCell dish PDLSCs sheet (Fig. 5F), and dissociated PDLSCs (Fig. 5I), but were irregular in the dissociated PDLSCs transplants. The percentage of periodontal bone was significantly higher in Vc-induced PDLSCs sheet and UpCell dish PDLSCs sheet transplant groups than in the dissociated PDLSCs transplant group. Furthermore, the percentage of periodontal bone was significantly higher in the Vc-induced PDLSCs sheet transplant group than in the UpCell dish PDLSCs sheet transplant group (Fig. 5J).

Vc induced human bone marrow mesenchymal cell sheet and umbilical cord mesenchymal cell sheet formation

After determining that Vc could induce the formation of PDLSCs sheet, we speculated that Vc might induce cell sheet formation for other MSCs. We isolated and cultured human BMMSCs and UCMSCs, and then induced them with $20\text{ }\mu\text{g/mL}$ Vc. Indeed, $20\text{ }\mu\text{g/mL}$ Vc can induce both BMMSCs and UCMSCs to form complete cell sheets; $20\text{ }\mu\text{g/mL}$ was the preferred concentration for the application (Fig. 6).

Discussion

Tissue engineering has long been thought to possess enormous potential; usually, isolated cell suspensions combined with bio-scaffolds were used for conventional applications. However, this procedure has had limited progress because of several complications. Therefore, "cell sheet engineering" was developed as an advanced approach, designed to avoid the shortcomings of traditional tissue engineering. When cultured cells are harvested as intact sheets along with their deposited ECM, they can be easily attached to host tissues, even wound sites, with minimal cell loss. They also maintain cell-to-cell and cell-to-ECM connections, which are generally required to re-create functional tissues. The use of cultured cell sheets also has the advantage of eliminating the use of scaffolds, prohibiting strong inflammatory responses that are induced when biodegradable scaffolds are degraded. "Cell sheet tissue engineering" has been used in many areas (Nishida et al., 2004; Shimizu et al., 2006; Ohashi et al., 2007), and has been beneficial for clinical applications, such as cell transplantation and tissue regeneration (Nishida et al., 2004). Since Okano and colleagues developed a temperature-responsive culture dish to harvest cultured cell sheets (Okano et al., 1993, 1995; Yang et al., 2007), several improvements have been made to harvest the living cell sheet more easily (Chen et al., 2006, 2007; Shimizu et al., 2009). In the latest study,

dexamethasone and Vc were used to create cell sheets to enhance bone formation (Nakamura et al., 2010). In this study, the optimal dose of Vc and the mechanism of Vc-induced cell sheet formation were not investigated.

In this study, we developed a novel, simple, and inexpensive method of harvesting MSCs. Vc was applied to induce cell sheet formation because of its properties of ECM and proliferation induction. According to our results, Vc induces PDLSCs to form cell sheets in a dose-dependent manner, and 20 $\mu\text{g/mL}$ Vc is the optimal concentration for complete cell sheets with a high level of success (10/10), more than the traditional method using a temperature-responsive culture dish (8/10). The Vc-induced PDLSCs sheet can be easily detached from culture dish and noninvasively harvested, not only with tight cell-to-cell junctions and deposited extracellular matrix (ECM), but also with microfilaments in the cytoplasm and exocytotic vesicles near the plasma membrane, which implies high cellular activity. Furthermore, Vc improved the proliferation ability of PDLSCs without causing a loss in the osteogenic differentiation capacity of the cells. These findings indicate that Vc may delay premature cell aging and inactivity, in addition to inducing cell sheet formation (Massip et al., 2010), inhibiting/repairing oxidative DNA damage, and preventing low-density lipoprotein oxidation by scavenging the reactive free radicals/oxidative species generated by various biological processes, as previously reported (Ji et al., 2010; Fraga et al., 1991; Halliwell, 2001; Naidu 2003).

In addition, more collagen and other ECM constituents were produced and preserved during Vc-induced cell sheet construction, including COL I, integrin $\beta 1$, and fibronectin, in accordance with previous studies (Prockop and Kivirikko, 1984; Murad et al., 1981). ECM is responsible for transmitting a wealth of chemical and mechanical signals that mediate key aspects of cellular physiology, such as adhesion, migration, proliferation, differentiation, and death, in addition to providing support for cellular tissues and physical sites for cellular attachment (Nelson and Bissell, 2006). The ECM also presents numerous signals to the cells that influence cellular activities and determine tissue structure and function. The preservation and generation of ECM are helpful for tissue regeneration. Previous studies have demonstrated that Vc may modulate Nanog expression and induce greater stemness by increasing histone demethylase activity (Cloos et al., 2008). Moreover, Vc inhibited differentiation and upregulated expression of pluripotency markers, such as Oct4 and Sox2 (Ji et al., 2010; Potdar et al., 2010). In addition Vc could accelerate gene expression changes and promote the transition of the pre-iPSC colonies into a fully reprogrammed state (Esteban et al., 2010). This greater "stemness" feature may prolong stem cell survival and support tissue regeneration.

In our study, telomerase activity and hTERT protein level in PDLSCs increased after Vc treatment. hTERT is one of key nuclear proteins which control DNA metabolism and proliferation (Bodnar et al., 1998). TERT was associated with highly osteogenic PDLSC clones (Sununliganon and Singhatanadgit, 2011). Furthermore, the combined ectopic expression of BMP4 and hTERT significantly enhanced the multipotent differentiation efficiency and capacity of human PDL fibroblasts, as shown by osteogenic, adipogenic and neurogenic differentiation in vitro, and cementum/PDL-like tissue regeneration in vivo (Mi et al., 2011). In consistent with previous reports, we found Vc increased hTERT activity, which might up-regulate COL I, integrin $\beta 1$, fibronectin, Oct4, Sox2, Nanog, RUNX2, ALP and OCN, and enhance the proliferation and differentiation efficiency of PDLSCs. At the same time, higher expression of the matrix proteins fibronectin and integrin $\beta 1$ was abrogated by the inhibition of telomerase. These results suggest that Vc-mediated PDLSCs sheet may have more potential for self-renewal and differentiation through telomerase activity. Indeed, transplantation in nude mice showed demonstrated more and better bone/cementum-like matrix formation with no remaining scaffold residue in Vc-mediated

PDLSCs sheet transplants compared with the cell sheets from a temperature-response culture dish. We also evaluated the ability of periodontal tissue to regenerate when supported by Vc-induced PDLSCs sheet in large animal-minipig. The anatomy, development, physiology, pathophysiology, and disease occurrence of minipig are similar to those of human's (Wang et al., 2007). Many studies have described the benefits of using minipigs as an ideal experimental animal model for many human diseases (Liu et al., 2008; Ding et al., 2010). In our study, we generated periodontitis lesions in the minipig, then tested the feasibility of using autologous PDLSCs sheet to repair the periodontitis induced bone defects. PDLSCs sheets appear to have a better capacity to form bone, cementum, and periodontal ligament compared with the UpCell dish PDLSCs sheet and dissociated PDLSCs, indicating that Vc-induced PDLSCs sheet could offer an optimal therapeutic approach for periodontal tissue regeneration.

Cell sheet-mediated tissue engineering on the basis of MSCs is not limited to periodontal tissues; it is also applicable to bone, corneal, myocardial, and other tissues (Nishida et al., 2004; Shimizu et al., 2006; Ohashi et al., 2007; Ding et al., 2010). We investigated whether this method was suitable for other tissue-derived MSCs. By adding 20 µg/mL Vc to the culture medium, well-constructed BMMSCs sheet and UCMSCs sheet were obtained. These results demonstrate that Vc could be used to induce the formation of different tissue-derived MSCs, and 20 µg/mL appears to be the preferred concentration. Taken together, we conclude that Vc is easily used to induce well-constructed and functional MSCs, and can improve tissue regeneration.

Conclusions

In this study, we found that Vc is capable of inducing telomerase activity in PDLSCs, leading to up-regulated expression of ECM and stem cell markers. We developed a new, simple, and practical approach to generate Vc-induced PDLSCs sheet in vitro. Implantation of PDLSCs sheet regenerated periodontal tissue in a miniature pig model. Vc-based cell sheet technique may offer an easy and practical tissue engineering approach.

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Fig. 1.

Vitamin C (Vc)-mediated periodontal ligament stem cell sheet. PDLSCs were induced by different concentrations of Vc (from 0 $\mu\text{g/mL}$ to 50.0 $\mu\text{g/mL}$) for 10 days. 0 $\mu\text{g/mL}$ (A), 5.0 $\mu\text{g/mL}$ (B), 10.0 $\mu\text{g/mL}$ (C) Vc did not induce PDLSCs to form a morphologically complete cell sheet. 20.0 $\mu\text{g/mL}$ (E) and 50.0 $\mu\text{g/mL}$ (F) Vc induced PDLSCs to form a perfect cell sheet, similar to the sheet obtained by the traditional temperature-responsive culture method (G). H&E staining revealed that the cell sheet was fragmented by 10.0 $\mu\text{g/mL}$ Vc (D), but the cell sheet is complete when 20.0 $\mu\text{g/mL}$ Vc is used (H); the whole PDLSCs sheet was two- or three-layered and the cells contacted each other tightly. Scale bars: 40 μm . Each experiment was conducted in triplicate.

Fig. 2.

Transmission electron microscopy (TEM) of a vitamin C (Vc)-induced periodontal ligament stem cell sheet. (A) Vc (20.0 $\mu\text{g/mL}$) was used to induce PDLSCs sheet formation and a monolayer cell sheet was formed, as observed by TEM. (B) Characteristic tight cell-to-cell connections were observed (arrows: junction sites). (C) The cytoplasm was microfilament-rich (ellipse), and exocytotic vesicles were observed near the plasma membrane (ellipse). (D) Endogenous extracellular matrix (ECM) (ellipse) was observed between cells.

Fig. 3.

The effect of vitamin C (Vc) on the periodontal ligament stem cell sheet. (A) Different concentrations of Vitamin improved the proliferation of PDLSCs. (B) Telomerase activity in PDLSCs increased gradually after treatment with ascorbic acid at a concentration of 20.0 µg/mL. HEK293T cells were used as a positive control and heat inactivated 293T cells were used as a negative control. (C) Human telomerase reverse transcriptase (hTERT) protein level gradually increased in the presence of ascorbic acid. (D) The relative mRNA expression of collagen type I (COL1), integrin β1, and fibronectin were higher in 20 µg/mL Vc-induced PDLSCs sheet compared with PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs. Oct4, Sox2, Nanog, RUNX2, ALP and OCN also increased in 20 µg/mL Vc-induced PDLSCs sheet compared with PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs; however, there was no significant difference between PDLSCs sheet obtained from an UpCell dish and dissociated PDLSCs (n = 5). (E) Alizarin red S staining suggested that 20 µg/mL Vc induced osteogenic differentiation. (F) The expression of matrix protein, fibronectin, and integrin β1 were enhanced by treatment with Vc, and this effect was abrogated by telomerase inhibition. * $P < 0.05$; ** $P < 0.01$; NS, no significance. Scale bars: 100 µm.

Fig. 4.

Periodontal ligament stem cell sheet-based cementum/ PDL-like tissue regeneration in nude mice. After 4 weeks of transplantation, PDLSCs differentiated into odontoblasts/ cementoblast-like cells (arrows) that formed much more regular bone/cementum-like matrix (rectangles) in the PDLSCs sheet transplants (A, B). While in dissociated PDLSCs transplants, PDLSCs formed less irregular bone/cementum-like matrix (rectangles) containing odontoblasts/cementoblast-like cells (arrows) and there was plenty of gelfoam scaffolding left (C). Goldner's trichrome staining revealed that bone/cementum-like matrix is blue (rectangles) (D, E, F). Picrosirius-red staining also revealed that there was much more condensed bone/cementum-like matrix generation in PDLSCs sheet transplants (rectangles) (G, H). The same polarized light view indicated that the condensed tissues were full of collagen type I (in red) and type III (in green) (rectangles) (J, K). Picrosirius-red staining showed that there were limited amounts of bone/cementum-like matrix regeneration (rectangles) remaining along the surface of gelfoam carriers in dissociated PDLSCs transplants (I); much less new tissue, including collagen type I (in red) and type III (in green) (rectangles); and plenty of remaining gelfoam carriers (no staining) were found when the samples were examined by polarized light (L). The percentages of bone/cementum-like matrix were significantly higher in the Vc-induced PDLSCs sheet and UpCell dish PDLSCs sheet transplant groups ($44.2\% \pm 5.9\%$ and $35.7\% \pm 4.6\%$, respectively) than in the dissociated PDLSCs transplant group ($12.5\% \pm 4.0\%$); the percentage of bone/cementum-like matrix was significantly higher in the Vc-induced PDLSCs sheet transplant group than in the UpCell dish PDLSCs sheet transplant group. Scale bars: 50 μ m.

Fig. 5. Regeneration of periodontitis defects mediated by periodontal ligament stem cell sheets in miniature pigs. H&E staining revealed new periodontal tissue regeneration in the periodontal defect area in the Vc-induced PDLSCs sheet group (A), UpCell dish PDLSCs sheet group (D) and dissociated PDLSCs group (G). The alveolar bone heights were much larger in Vc-induced PDLSCs sheet (A) and UpCell dish PDLSCs sheet (D) groups than in the dissociated PDLSCs group (G). Much thicker sulcular epithelia were evident in the dissociated PDLSCs group (H) compared with the Vc-induced PDLSCs sheet (B) and UpCell dish PDLSCs sheet (E) groups. Sharpy's fibers formed in the Vc-induced PDLSCs sheet group (C), UpCell dish PDLSCs sheet group (F), and dissociated PDLSCs group (I);

however, Sharpy's fibers were irregular in the dissociated PDLSCs group. (J) The percentage of periodontal bone was significantly higher in Vc-induced PDLSCs sheet and UpCell dish PDLSCs sheet groups ($55.3\% \pm 5.1\%$ and $43.6\% \pm 3.3\%$, respectively) than in the dissociated PDLSCs group ($31.4\% \pm 4.5\%$). Abbreviations: SE, sulcular epithelium; CEJ, cemento-enamel junction; HAB, height of alveolar bone; D, dentin; C, cementum; PDL, periodontal ligament; B, bone.

Fig. 6. Vitamin C (Vc) induced complete cell sheet formation of bone marrow mesenchymal stem cells (BMMSCs) and umbilical cord mesenchymal stem cells (UCMSCs). Vitamin C (Vc, 20.0 $\mu\text{g/mL}$) was used to induce sheets of two other mesenchymal stem cell types for 10 days. Morphological observation indicated that complete sheets of BMMSCs (A) and UCMSCs (C) were formed. H&E staining revealed the two- or three-layered, uniformly spread, two-dimensional tissue structure in whole cell sheets of BMMSCs (B) and UCMSCs (D). Scale bars: 20 μm . All experiments were conducted in triplicate.

Table 1

List of primers used in real time PCR

Primer ID	Accession No.	Sequence(5'-3')	Expect size (bp)
Collagen Type I-F	<i>NM_000088</i>	GCTGATGATGCCAATGTGGTT	155
Collagen Type I-R		CCAGTCAGAGTGGCACATCTTG	
Integrin β 1-F	NM_002211	GTGAGTGCAACCCCAACTACACT	219
Integrin β 1-R		AAGGCTCTGCACTGAACACATTC	
Fibronectin-F	<i>NM_212476</i>	CACCCAATTCTTGCTGGTATC	162
Fibronectin-R		TATTCGGTTCCCGTTCCA	
Oct4-F	NM_002701	CACTGTACTCTCGGTCCCTTTC	147
Oct4-R		CAGGCACCTCAGTTTGAATGC	
Sox2-F	<i>NM_003106</i>	CCAGCTCGCAGACCTACATGA	153
Sox2-R		CTGGAGTGGGAGGAAGAGGTAAC	
Nanog-F	<i>NM_024865.2</i>	CCTTGGCTGCCGTCTCTGGCT	150
Nanog-R		AGCAAAGCCTCCCAATCCCAAACAA	
Runx2-F	NM_001024630	GTTCACCTTGACCATAACCGT	198
Runx2-R		GGGACACCTACTCTCATACTGG	
ALP-F	<i>NM_002211</i>	TCCATCTGTAAAGGGCGGTAAT	110
ALP-R		AATACCAGCTACGCTGCATCAAG	
OCN-F	NM_199173	AATCCGGACTGTGACGAGTTG	121
OCN-R		CAGCAGAGCGACACCCTAGAC	
β -actin-F	NM_001101	TGCCGACAGGATGCAGAAG	147
β -actin-R		GCTGATCCACATCTGCTGGAA	