Simvastatin alters fibroblastic cell responses involved in tissue repair


Background and Objective: Statins have been used to control hypercholesterolemia. However, these drugs also exert pleiotropic effects that include the modulation of inflammation and cell signaling. The present study has analyzed the effects of simvastatin on several cell responses involved in tissue repair, including cell adhesion, cell migration and invasion, actin cytoskeleton remodeling and cell viability.

Material and Methods: Primary cultures of gingival fibroblasts were stimulated with simvastatin. Cell adhesion was evaluated using a colorimetric assay. Cell spreading was evaluated microscopically. Cell migration and invasion were assessed using a scratch wound-healing assay and a bicameral cell culture system, respectively. Changes in actin cytoskeleton and focal adhesion assembly were evaluated through immunofluorescence for actin, vinculin and active β1 integrin. Rac activation was evaluated by means of a pull-down assay. Cell viability was assessed using a colorimetric assay that determines mitochondrial functionality. Data analysis was performed using the Mann–Whitney U-test.

Results: Simvastatin diminished cell adhesion and spreading over a fibronectin matrix. It also altered the closure of scratch wounds induced on cell monolayers and cell invasion through a Transwell system. Simvastatin-treated cells displayed an altered lamellipodia with poorly developed focal adhesion contacts and reduced levels of β1 integrin activation. During cell spreading, simvastatin diminished Rac activation.

Conclusion: The present study shows that simvastatin may alter cell migration by disrupting the cell signaling networks that regulate the actin cytoskeleton dynamics. This mechanism may affect the response of gingival mesenchymal cells during wound healing.

Statins are widely used medications to prevent cardiovascular events in patients with hypercholesterolemia (1,2). These drugs are able to reduce the biosynthesis of the cholesterol precursor L-mevalonate through inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (3). Besides this effect of statins, several studies have documented that these drugs may also modulate the inflammatory response by a mechanism that is independent from cholesterol reduction (4,5).

Fibroblasts constitute the predominant cell phenotype in gingival tissues and play important roles in both the preservation of tissue homeostasis and the response to tissue injury and infection (6). During tissue repair, fibroblasts must attach to the provisional extracellular matrix (ECM), predominantly composed of a fibrin–fibronectin network (7). Following injury, resident cells are dislodged from their tissue environment and are activated to migrate into the wound site,
divide, differentiate and produce a new ECM. Therefore, cell adhesion and migration are important components of a physiological response that must be preserved to permit the normal progression of the wound-healing process (8). Cell adhesion is critical for cell migration to occur, and this process is mediated by the continuous formation of cytoplasmic protrusions at the leading edge of the cell and controlled retraction of adhesive contacts at its rear (8). Focal adhesions provide dynamic links between the actin cytoskeleton and the ECM, and β1 integrins and vinculin are considered key proteins that mediate this functional connection during migration and adhesion (9).

The Rho GTPase family currently consists of seven distinct proteins involved in critical cell functions, such as actin cytoskeleton remodeling, cell growth, cell signaling and migration (10). Small GTPases of the Rho family are pivotal regulators of several signaling networks and, like other regulatory GTP-binding proteins, Rho-GTPases act as molecular switches by cycling between GDP- and GTP-bound states (11). Several studies have demonstrated that statins prevent isoprenylation of small G-proteins and thereby alter their subsequent function (10). Both the homeostasis of gingival connective tissues and the evolution of the wound-healing process may be affected by factors regulating the development of the actin cytoskeleton, the formation of cell–matrix adhesive interactions and the ability of cells to migrate. Therefore, factors that may affect these cellular functions might compromise the normal response of gingival tissues to either traumatic or infectious challenges.

Although several studies have been performed to determine the effects of statins on periodontal disease prevalence or severity, conflicting results have been reported showing: (i) no association between drug intake and tooth loss (12); (ii) a protective effect for statin medication on periodontal tissue damage (13); or even (iii) a negative effect for intake of statins on the prevalence of periodontal pockets (14). This discrepancy clearly indicates that further studies are needed to better understand the effects of statins on gingival tissues.

Considering the described effects of statins on cell signaling and their still poorly characterized role on gingival tissue physiology, the present study was performed to identify whether statins may alter a repertoire of cellular activities involved in gingival tissue repair.

Material and methods

Cell culture

Primary cultures of human gingival fibroblasts were established by the explant method (15). Tissue explants were obtained from clinically healthy or severe gingival connective tissue of six individuals (two women and four men; age range 18–32 years) undergoing extraction of third molars at a private dental practice in Santiago, Chile between April and November 2008. Tissue samples were harvested with the informed consent of the patients. The protocol for obtaining tissue was approved by the Ethical Committee, Faculty of Medicine, Pontificia Universidad Católica de Chile. Patients reported no relevant pre-existing medical or drug histories during the last 6 months. Cells were cultured in α-minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA), 100 mg/mL penicillin (Sigma, St Louis, MO, USA), 100 mg/mL streptomycin (Sigma) and 50 mg/mL gentamicin (Sigma) at 37°C in an atmosphere of air containing 5% CO2. All experiments were performed using cells expanded between passages four and 10.

Cell adhesion assay

For cell adhesion, non-tissue-culture 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 μg/mL fibronectin (Calbiochem, San Diego, CA, USA) in phosphate-buffered saline (PBS). Nonspecific binding sites were blocked by denatured bovine serum albumin (Rockland Immunochemicals, Gilbertsville, PA, USA). Cells were detached from the cell culture plates by a brief exposure to trypsin/EDTA (Hyclone Laboratories Inc.) and counted. One thousand cells were seeded over fibronectin-coated plates for 7 min. Cell adhesion was stopped by pouring off the medium. Cells were fixed with methanol for 10 min at room temperature. Absorbance at 570 nm was analyzed on a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT, USA) as described previously (16). All assays were performed in quadruplicate in three separate sets of experiments.

Cell-spreading assay

Cells were detached from cell culture plates by a brief exposure to trypsin/EDTA (Hyclone Laboratories Inc.) and counted. One thousand cells were stimulated with 1, 5 or 10 μM simvastatin (Merck) for 1 h in the absence of FBS. Afterwards, cells were seeded onto fibronectin-coated plates for 45 min in α-MEM plus 1% FBS. Cells were fixed with methanol for 2 min and incubated with 0.2% crystal violet for 5 min. After removing excess dye, cells were solubilized in 0.1 M NaH2PO4 in 50% methanol for 10 min at room temperature. Absorbance at 570 nm was analyzed on a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT, USA) as described previously (16). All assays were performed in quadruplicate in three separate sets of experiments.

Cell-invasion assay

The ability of gingival fibroblasts to migrate was assayed using Transwell chambers (BD Bioscience USA, Bedford, MA, USA) with 8.0-μm-pore polycarbonate filters (Collaborative Research, Bedford, MA, USA) coated with 10 μg/mL of a reconstituted...
basement membrane (Matrigel; Costar, Cambridge, MA, USA) on the upper side of the filter. Cells were previously exposed to different simvastatin concentrations for 16 h in the absence of FBS, then suspended in serum-free medium and seeded on the upper compartment of the chamber. Control cells were stimulated with dimethyl sulfoxide. To stimulate cell migration, 10% FBS was added to the lower compartment of the chamber. Migration was allowed to occur for 16 h. Following the removal of the noninvading cells from the upper surface of the reconstituted basement membrane with a cotton swab, the invading cells were fixed and stained with 0.2% crystal violet. Cell migration was evaluated by counting five (≥20) fields per chamber as described previously (16). All assays were performed in quadruplicate in three separate sets of experiments.

Cell viability assay (MTT assay)

The MTT™ (Roche Diagnostics GmbH, Mannheim, Germany) proliferation assay was used to assess the effect of simvastatin on cell viability. This is a colorimetric assay where the amount of color produced is directly proportional to the number of viable cells. Cells were seeded into 96-well plates (Nunc) and allowed to attach overnight in the presence of FBS. Cells were then exposed to different simvastatin concentrations in the presence of 1% FBS for 24. The MTT labeling reagent was then added to each well and incubated for 4 h. After this, cells were washed with PBS and a solubilization solution was added. The plates were then incubated overnight and subsequently read at 570 nm using a microplate reader.

Wound-closure assay

Gingival fibroblasts were seeded on 24-well plates (Nunc). When the cells achieved 90% confluence, cell cultures were scratched with a 200 μL sterile pipette tip and washed with PBS to remove detached cells and debris. Cells were then incubated in medium containing 1% FBS and different simvastatin concentrations. Dimethyl sulfoxide was added to control cells. After 16 h, cells were fixed with methanol for 2 min, and incubated with 0.2% crystal violet for 5 min. This experiment was performed on three separate occasions. Images of each wound were captured with a digital camera (Nikon Coolpix 4500; Nikon, Tokyo, Japan) through an inverted microscope (Zeiss Axiosvert 25; Zeiss, Bernried, Germany). Cell layer margins were outlined using the polygonal selection tool of AdobePhotoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). The ratio of wound closure was determined using MS Excel 2003 (Microsoft, Redmond, WA, USA). These assays were performed in three separate sets of experiments.

Immunofluorescence of gingival fibroblasts

The distributions of vinculin, active β1 integrin and actin filaments were evaluated through immunofluorescence. In each set of experimental conditions, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.25% Triton X-100 for 4 min. Coverslips were washed three times with staining buffer (×1 PBS, 0.1% sodium azide and 1% FBS). Primary antibodies diluted in staining buffer were used at a dilution of 1:100 for anti-vinculin (Sigma) and 1:100 for anti-β1 integrin (Chemicon, Temecula, CA, USA). The antigen–antibody complex was washed and incubated with fluorescein 5-isothiocyanate-conjugated anti-mouse monoclonal antibody (Invitrogen Molecular Probes, Carlsbad, CA, USA). F-actin was stained with phalloidin–rhodamine (Invitrogen). Fluorescence images were examined with a microscope (Zeiss Axioplan) and photographed using a ×63 immersion objective and a digital camera (Zeiss). These experiments were performed on three separate occasions.

Pull-down assay for Rac

Serum-starved human gingival fibroblasts were detached and stimulated under suspension with 5 μM simvastatin in the presence of 1% FBS for 1 h. Afterwards, cells were adhered onto fibronectin-coated plates for 90 min in α-MEM plus 1% FBS supplemented with 5 μM simvastatin or the drug vehicle for 90 min. Cells were washed once with ice-cold PBS and lysed with 50 mM Tris, pH 7.6, 0.5 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 10 μg/mL of each aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 19,000 g for 10 min, and the supernatant was incubated on a roller for 1 h with 20 μg of glutathione transferase protein binding domain (GST-PBD) [GST fusion protein containing the Rac-binding domain (amino acids 67–150)] of p21 activated kinase 1 (Cytoskeleton, Denver, CO, USA). Samples were washed three times with lysis buffer and bound proteins eluted by boiling in sample buffer and then immunoblotted against Rac with monoclonal antibodies (Cytoskeleton). Whole-cell lysates were also immunoblotted for Rac as a loading control.

Statistical analysis

Statistical analysis was performed for cell adhesion, wound closure, cell viability and cell-spreading assays using the Mann–Whitney U-test. Cell invasion (Transwell experiment) was also analyzed using a simple regression analysis. All these analyses were performed using the SPSS software for Windows (version 16.0.2; SPSS Inc., Chicago, IL, USA).

Results

Simvastatin affects cell adhesion and spreading in gingival fibroblasts

Cell–matrix interactions are significant events during tissue repair. To evaluate whether simvastatin may modulate these responses, serum-starved gingival fibroblasts were exposed to a range of simvastatin concentrations (5, 10, 20 and 30 μM) for 16 h. After this, cells were detached from culture plates and seeded over fibronectin-coated dishes for 7 min. As shown in Fig. 1A, simvastatin induced a dose-dependent
reduction in cell adhesion that reached statistical significance at 20 and 30 μM. To identify whether simvastatin may affect the ability of cells to spread over a fibronectin matrix, suspended gingival fibroblasts were exposed to 1, 5 or 10 μM simvastatin for 1 h in the absence of FBS and seeded on fibronectin-coated plates for 45 min in α-MEM supplemented with 1% FBS. Visualization of crystal violet-stained cells revealed that simvastatin-treated gingival fibroblasts had fewer cell extensions spreading over the fibronectin-coated culture dishes (Fig. 1B). Quantification of this experiment resulted in a statistically significant reduction in the proportion of spread cells when exposed to simvastatin (Fig. 1C).

Simvastatin alters the development of cell extensions and Rac activation in gingival fibroblasts

To characterize the morphological alterations observed in cells exposed to simvastatin, gingival fibroblasts were exposed to simvastatin or its vehicle and seeded over fibronectin-coated coverslips for 90 min. Immunofluorescence for the focal adhesion protein vinculin and actin filaments demonstrated that simvastatin-exposed cells had thinner cell extensions and fewer focal adhesions when compared with control cells, which displayed a wide lamellipodium with several vinculin-stained focal contacts extending over the fibronectin matrix (Fig. 2A). Considering the role exerted by Rac in lamellipodium formation (17), we analyzed the activation of this GTPase in the presence of simvastatin. To this end, serum-starved gingival fibroblasts were exposed under suspension to 5 μM simvastatin for 1 h. After this, cells were plated over fibronectin-coated dishes in α-MEM supplemented with 1% FBS, preserving the stimulus with 5 μM simvastatin. After 90 min, a pull-down experiment was performed to identify Rac activation during the spreading of cells over the fibronectin matrix. As shown in Fig. 2B, simvastatin-exposed cells had lower Rac-GTP levels when compared with vehicle-stimulated cells.
Simvastatin retards wound closure in gingival fibroblasts

The effect of simvastatin on cell migration was evaluated using a scratch wound-healing assay. Cells were plated on fibronectin-coated dishes and exposed to varying concentrations of simvastatin in α-MEM supplemented with 1% FBS. After 16 h, we observed a significant delay in wound closure in the presence of simvastatin (Fig. 3A). This experiment was performed on three separate occasions, and similar results were obtained. Quantification of this experiment demonstrated a dose-dependent decrease in cell migration that reached statistical significance at 1 μM simvastatin (Fig. 3B).

Simvastatin alters cell invasion in gingival fibroblasts

We further evaluated the ability of simvastatin to modulate cell migration and invasion through a reconstituted ECM using a bicameral cell culture system. Gingival fibroblasts were exposed to different concentrations of simvastatin overnight and then seeded on the upper side of a Transwell chamber. Matrigel was used as a barrier to evaluate the invasive potential of the cells. As revealed in Fig. 4A, fewer cells were observed in the lower side of the filters of cells exposed to increasing concentrations of simvastatin. Quantification of these results demonstrated a dose-dependent and statistically significant reduction in the invasive capacity of simvastatin-exposed cells (Fig. 4B).

Effect of simvastatin on cell viability

To identify the effect of simvastatin on cell viability, cells were exposed to increasing concentrations of this drug (0.1–50 μM) or dimethyl sulfoxide in the presence of 1% FBS. Cell viability was evaluated at 24 h through the MTT assay. Cell viability was not compromised until simvastatin concentrations reached 50 μM (Fig. 5).

Discussion

The present study has evaluated the effects of simvastatin on several cell responses involved in tissue repair. Our results show that simvastatin altered the ability of fibroblasts to adhere and spread over a fibronectin matrix, inhibited cell migration in scratch wound-healing assays and inhibited the capacity of cells to invade a reconstituted ECM (cell-invasion assay). In the presence of simvastatin, cells located at the migration front showed poorly developed lamellipodia with few focal adhesion contacts, probably due to lower levels of active Rac. These results suggest a mechanism through which simvastatin may alter adhesion, spreading and cell migration and probably modify the locomotion of mesenchymal cells into wounded areas of the gingival tissues.

One of the key steps of wound healing is the migration of mesenchymal cells into a fibrin–fibronectin clot during the development of granulation tissue (7). Cell migration can be divided into the following four separate steps: lamellipodium extension, formation of new adhesions, cell body contraction and tail detachment (17). In our initial experiments, we observed that simvastatin was able to diminish cell adhesion over a fibronectin matrix. We further observed that cell migration was significantly retarded by simvastatin using the scratch wound-healing assay. A remarkable finding is that cell adhesion was affected by 20 μM simvastatin, while cell migration and spreading were altered in the presence of 1 μM simvastatin. This

![Simvastatin alters cell morphology and Rac activation.](link)
finding is in accordance with the morphological changes visualized by immunofluorescence of spreading and migrating cells that demonstrated a reduced lamellipodia when compared with control cells. Cell migration is regulated by Rho proteins that cycle between an active GTP-bound conformation and an inactive GDP-bound state (17). Rac is a key regulator of cell migration because of its ability to stimulate lamellipodium extension (19). Meanwhile, Rho is required for focal adhesion assembly and polymerization of actin stress fibers (17). It is interesting to note that cell migration and spreading is significantly retarded in Rac1-deficient fibroblasts and that tissue healing is also retarded in Rac-deficient mice (20). Rac is also necessary to drive the chemotactic response of fibroblasts induced by platelet-derived growth factor (21) and for cell invasion of a reconstituted ECM (22).

At a molecular level, Rac stimulates formation of lamellipodia through activation of the actin-nucleating activity of the actin-related proteins 2/3 (23). These mechanisms connecting Rac, lamellipodium formation and cell migration reinforce the findings of the present study, in which simvastatin-exposed cells showed reduced levels of active Rac during cell spreading and a retarded migratory response. It is important to mention that the effect of statins on Rho GTPases is a controversial issue that seems to depend on the cell type under study and drug dosage (10,24). In a monocyte cell line (THP-1) both simvastatin and lovastatin stimulated the loading of Rac and RhoA by GTP. However, these species were functionally inactive (10).

Other studies have reported that statins inhibit GTP loading of RhoA in human monocytes (2) and MT-2 cell lines (25). Our results show a novel mechanism of action for simvastatin that may affect the activation of Rac during cell spreading and the development of lamellipodia. This effect may finally have an impact on cell locomotion in gingival fibroblasts.

The three-dimensional cell-invasion assay evaluates the ability of cells to pass through a reconstituted basement membrane. This model resembles the physiological environment, in which mesenchymal cells migrate and populate the granulation tissue during wound healing. In this assay, we observed that simvastatin, at a concentration as low as
M, was able to inhibit cell migration and invasion of the Matrigel matrix. To pass through a three-dimensional matrix, cells must adjust their actin cytoskeleton towards a flexible form, extending a front lamellipodium and degrading the extracellular environment to release cell-matrix contacts (9). Previous studies have identified that statins may reduce production of MMPs in several cell types, including monocytes and smooth muscle cells (26,27). Although we did not evaluate whether simvastatin was able to modulate the expression or activity of MMPs, it is possible to propose that MMPs might be one of the targets of statins that should be explored in future studies.

The cell viability assays allowed us to validate our results, since they showed that cell death was not affecting the outcome of the experiments performed in this study. However, it is important to note that exposure of cells to 50 µM simvastatin for 24 h has an impact on cell viability. In fact, statins may induce apoptosis in endothelial cells and lung fibroblasts (28,29). Future studies should explore the specific role of statins on cell death/viability in gingival mesenchymal cells.

The primary action of statins is the inhibition of HMG-CoA reductase, a pharmacological activity that finally reduces de novo synthesis of cholesterol (3). Besides this effect, several studies have demonstrated that statins may affect the activity of small G-proteins of the Ras superfamily because of their isoprenylation (10). Small G-proteins are involved in several cell functions, including the regulation of the actin cytoskeleton, cell migration, intracellular traffic and cell survival/proliferation (10). The Rho GTPases also play a role in transduction of inflammatory signals, such as nuclear factor-κB and c-Jun NH2-terminal kinase pathways (30). Therefore, it is possible to propose that simvastatin may affect several aspects of gingival tissues, including the evolution of inflammation and critical cell functions involved in tissue repair, such as cell adhesion, actin cytoskeleton dynamics, cell migration and survival. Periodontal disease is an inflammatory disorder initiated by pathogens that colonize the subgingival environment. The evolution of periodontal disease may be interpreted as a cyclic process characterized by alternating periods of tissue destruction followed by repair (31). Therefore, it is possible to propose that factors that modulate gingival tissue repair may affect the final evolution of disease. In oral epithelial cell lines, simvastatin reduces the production of interleukin-6 and interleukin-8 (32). In mononuclear cells, simvastatin may also reduce the production of MMP-1 (26). Therefore, it has been suggested that statins might play a protective role in periodontal tissues through inhibition of inflammation. However, several discrepant studies have been unable to demonstrate either a protective or a
negative role for statins on periodontal health (12–14). Our results show that simvastatin may alter important cellular activities that may diminish the ability of mesenchymal cells to restore the damaged tissues. In accordance with this concept, long-term statin administration has been associated with muscle damage (33,34). Considering these findings, future clinical studies should explore the role of statins in gingival tissue physiology and repair.

Our results show that cell functions such as cell spreading and migration were affected by simvastatin concentrations ranging between 1 and 5 μM. Although these concentrations seem to be rather low, we could not identify studies reporting the serum or tissue levels of simvastatin. Therefore, clinical or animal studies designed to identify the levels achieved by these drugs in tissues are fundamental to better define the role of statins in periodontal inflammation and healing.

Within the limits of the present study, our observations show that simvastatin exerts a detrimental effect in critical cell functions in gingival fibroblasts that may affect the evolution of tissue repair.

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