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Chitosan and platelet-derived growth factor synergistically stimulate cell proliferation in gingival fibroblasts

D. Silva[1], R. Arancibia[1], C. Tapia[2], C. Acuña-Rougier[2], M. Diaz-Dosque[3], M. Cáceres[1], J. Martínez[4], P. C. Smith[1]

[1]Dentistry Academic Unit, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Faculty of Odontology, University of Chile, Santiago, Chile, [3]Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile and [4]Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

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Background and Objective: Chitosan is a naturally derived polymer that may be applied in periodontal therapy for tissue-reconstruction purposes. Previous studies have shown that chitosan may stimulate tissue healing. However, reports exploring the cellular responses stimulated by chitosan are lacking. In the present study we analyzed whether chitosan may promote cell proliferation in primary cultures of human gingival fibroblasts.

Material and Methods: Chitosan particles were generated, and their size, zeta potential and morphology were characterized using transmission and scanning electron microscopy and zetasizer analysis. The biocompatibility of chitosan particles was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell-viability assay and by detecting the release of lactate dehydrogenase into the cell-culture medium. The total number of cells was estimated by staining with crystal violet followed by measurement of the absorbance at 560 nm on a microplate reader. Cell proliferation was studied by detecting proliferating cell nuclear antigen protein levels, immunofluorescence for Ki67 and incorporation of 5′-bromo-2′-deoxyuridine.

Results: The sizes of the chitosan particles generated were in the micrometer and nanometer ranges. Cell viability was increased in the presence of chitosan. Moreover, the combination of chitosan and platelet-derived growth factor (PDGF-BB) potently stimulated cell viability, cell proliferation and activation of the ERK1/2 pathway involved in cell proliferation.

Conclusions: The present study shows that chitosan is well tolerated by gingival fibroblasts and is able to stimulate cell proliferation through the ERK1/2 signaling pathway. A synergistic response between chitosan and growth factors (such as PDGF-BB) may stimulate cell proliferation in gingival fibroblasts exposed to this biomaterial.

Patricio C. Smith, DDS, PhD, School of Dentistry, Faculty of Medicine, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago 8330024, Chile
Tel: + 56 02 2 354 3408
Fax: +56 02 2 632 2802
e-mail: psmith@med.puc.cl

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Wound healing is characterized by a series of orchestrated phases: (i) hemostasia; (ii) inflammation; (iii) granulation tissue; and (iv) tissue remodeling (1). During these cellular events, and particularly during the granulation tissue phase, the population of gingival fibroblasts is stimulated to proliferate and several growth factors may promote cell division during these events (2). A critical issue in periodontology is the reconstruction of injured gingival and periodontal tissues (3). To achieve this, cell–biomaterial interactions are critically important to drive

cell and tissue responses. Therefore, a desirable effect of the biomaterials present in membranes used for tissue regeneration is the promotion of cell proliferation.

Chitin is a linear, unbranched polysaccharide that originates from the shells of crustaceans (4). Its deacetylated derivative, known as chitosan, has been shown to possess antimicrobial and drug-delivery properties (5). Therefore, several studies have proposed the use of chitosan to stimulate wound healing (6,7). Some of these applications have exploited the ability of chitosan to act as a drug-delivery system for antibiotics and recombinant growth factors (8–10). Chitosan membranes have been used to promote skin, tendon, bone and periodontal wound healing, and significant histological evidence of tissue regeneration has been reported (11,12). However, the precise role of chitosan in modulating cell-regenerative responses in periodontal cells remains to be studied. In skin fibroblasts, Howling *et al.* (13) described that chitosan may stimulate cell proliferation. However, this study also indicated a great variability in this response, as chitosan was also able to inhibit proliferation of skin keratinocytes (13). It is not known whether chitosan may modulate the proliferation of gingival fibroblasts, which display several differences compared with fibroblasts that originate from distinct tissues, including skin (14,15).

Platelet-derived growth factor (PDGF) is a polypeptide released by platelets after degranulation during blood clotting (16) and is able to stimulate cell proliferation in mesenchymal cells (17) and gingival fibroblasts (18). During wound healing, fibroblasts interact with growth factors and a great number of genes involved in cell proliferation are transcribed (19). *In vivo*, cells are probably exposed to biomaterials (such as chitosan) and to growth factors, so it is important to clarify the response of cells to both agents.

In the present study we assessed, using primary cultures of human gingival fibroblasts, whether chitosan particles are able to stimulate cell proliferation. We also explored whether chitosan collaborates with other growth factors, such as PDGF, to exert this mitogenic effect. This information may be important when considering the use of chitosan particles in different modalities of periodontal therapy or reconstruction.

Material and methods

Cell culture

Primary cultures of human gingival fibroblasts were produced using the explant method. Tissue explants were obtained from the retromolar tissue of four individuals undergoing the extraction of third molars at a private dental practice in Santiago, Chile. 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. No history of inflammation of the retromolar tissue was reported. Patients did not report relevant pre-existing medical or drug histories during the last 6 mo. Cells were cultured in α-Minimum Essential Medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA) and Normocin™ (Invivogen, San Diego, CA, USA) at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells expanded between passages 4 and 10.

Preparation of chitosan nanoparticles

Nanoparticles were produced based on the ionic gelation of tripolyphosphate and low-molecular-weight chitosan (Sigma, St Louis, MO, USA), as previously described (20). Nanoparticles were spontaneously obtained upon the addition of 1.2 mL (0.84 mg/mL, weight by volume) of a tripolyphosphate aqueous basic solution to 3 mL of the chitosan acidic solution (2 mg/mL, weight by volume) under magnetic stirring at room temperature. The obtained mixture was centrifuged at 19,000 *g* for 30 min, lyophilized, crushed with a mortar and sieved through a 400 mesh particle size.

Morphology of chitosan particles

The surface morphology of the chitosan particles was examined using scanning electron microscopy (Zeiss DMS 409; Zeiss, Zeiss Axioplan, Göttingen, Germany) by placing the sample directly onto the stub. Particle morphology was also analyzed using transmission electron microscopy (Zeiss EM 109; Zeiss). Chitosan was suspended in deionized water and then a drop of the suspension was placed on a copper grid. After 3 min the drop was removed using filter paper and 5% aqueous uranyl acetate was added over the grid. After 5 min the uranyl acetate was removed from the grid using filter paper and the grid was then viewed using a transmission electron microscope.

Analysis of particle size and zeta potential— Average particle size and zeta potential were measured using the Malvern Metasizer equipment (Spectris, Surrey, UK).

Western blotting

Cells were lysed with buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) at 4°C. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane by electroblotting (PerkinElmer Life Sciences, Boston, MA, USA). The membranes were exposed to primary antibodies against proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1/2 (Santa Cruz Biotechnology) and phospho-ERK1/2 (Santa Cruz Biotechnology) and then to secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). These membranes were then developed. The resulting bands were digitalized and

densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). These experiments were performed on three separate occasions.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to assess cell viability at different time points. This is a colorimetric method used to determine the number of viable cells, and the amount of color produced is directly proportional to the number of viable cells. Cells were seeded into 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and were allowed to attach overnight in the presence of FBS (Hyclone Laboratories Inc., Logan, UT, USA). The cells were then exposed to different concentrations of chitosan and/or to 1 ng/mL of PDGF for 24, 48 and 72 h. Twenty-microlitres of MTS labeling reagent was then added to each well and incubated for 4 h, and then the absorbance of each well was read at 492 nm using a microplate reader (ThermoPlate TP-Reader NM; Thermo Fisher Scientific, Rockford, IL, USA). These experiments were performed on three separate occasions.

Lactate dehydrogenase release assessment (lactate dehydrogenase assay)

Cells were seeded into 96-well plates (Orange Scientific) and were allowed to attach overnight in the presence of FBS (Hyclone Laboratories). The cells were then exposed to different concentrations of chitosan for 24, 48 and 72 h. Cell-culture medium was collected to assess lactate dehydrogenase (LDH) release following cell death. A lysis solution consisting of 1% Triton X-100 was used as a positive control of LDH release from cell cultures. Fifty microlitres of medium was then transferred to a 96-well plate and mixed with 50 μL of the LDH substrate mix (Promega Cytotox 96 nonradioactive cytotoxicity assay). Then, the plate was covered and incubated for 30 min at room temperature and protected from light. Fifty microlitres of stop solution was then added and the absorbance at 490 nm was measured. These experiments were performed on three separate occasions.

Immunofluorescence

Gingival fibroblasts were seeded over coverslips (25 000 cells per coverslip). Cells were stimulated with chitosan or PDGF-BB, or with the combination of both agents, for 48 h. Then, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.25% Triton X-100 for 4 min and washed three times with staining buffer (phosphate-buffered saline containing 0.1% sodium azide and 1% fetal bovine serum). Primary antibodies for KI67 (Abcam, Cambridge, MA, USA) and BrdU (Dako, Glostrup, Denmark) were diluted at 1:100 in staining buffer. The antigen–antibody complex was washed and incubated with a secondary antibody conjugated to fluorescein 5-isothiocyanate (Invitrogen Molecular Probes, Carlsbad, CA, USA). Nuclei were stained using Hoescht 33342 (Invitrogen Molecular Probes). Fluorescence images were examined using a microscope (Zeiss Axioplan, Göttingen, Germany) and photographed using a digital camera (Zeiss). To quantify the number of cells positive for each cell marker (Ki67 or BrdU) a total of six fields per coverslip were evaluated. These experiments were performed on three separate occasions. BrdU immunostaining was performed as previously reported (21).

Crystal violet staining and quantification— Gingival fibroblasts were seeded on 96-well plates (5000 cells per well). After exposure to the appropriate experimental stimuli, the cells were fixed and stained with 0.2% crystal violet for 5 min. After removal of excess dye, the cells were solubilized in 0.1 M NaH_2PO_4 in 50% methanol for 10 min at room temperature. The absorbance at 560 nm was read using a microplate reader, as previously described (22). These experiments were performed on three separate occasions.

Statistical analysis

For statistical comparison between conditions under observation, analysis of variance followed by Scheffé's post-hoc test was used. The software SPSS for Windows was used (version 16.0.2; SPSS Inc., Chicago, IL, USA).

Results

Evaluation of size and morphology of chitosan particles

Using scanning electron microscopy and transmission electron microscopy we evaluated the morphology of the chitosan particles. We observed a wide variation in the size of the particles under study. At low magnification (50 ×) we were able to identify a group of particles that were smaller than 200 μm in size (Fig. 1A). At higher magnifications (5000 and 10 000 ×) we observed a group of smaller particles that, in most cases, were attached to the surface of the larger particles shown in Fig. 1A (Fig. 1B and 1C). Using transmission electron microscopy we observed that many of the smaller particles had a rounded morphology, as shown in Fig. 1D. Using the Malvern Metasizer we determined that the average size of the particles was 221.3 nm. The zeta potential of the chitosan particles, measured using the Malvern Metasizer, was $+35.2 \pm 8.2$ mV, indicating that the particles had a positive charge and displayed a moderate stability in water suspension (23,24).

Biocompatibility and cell proliferation induced by chitosan particles

We then evaluated whether chitosan particles were able to modify cell viability. Serum-starved human gingival fibroblasts were exposed to 100, 300 and 600 μg/mL of chitosan particles. After 48 h of cell culture we evaluated LDH release into the cell-culture medium as an indicator of cell injury. As observed in Fig. 2, LDH

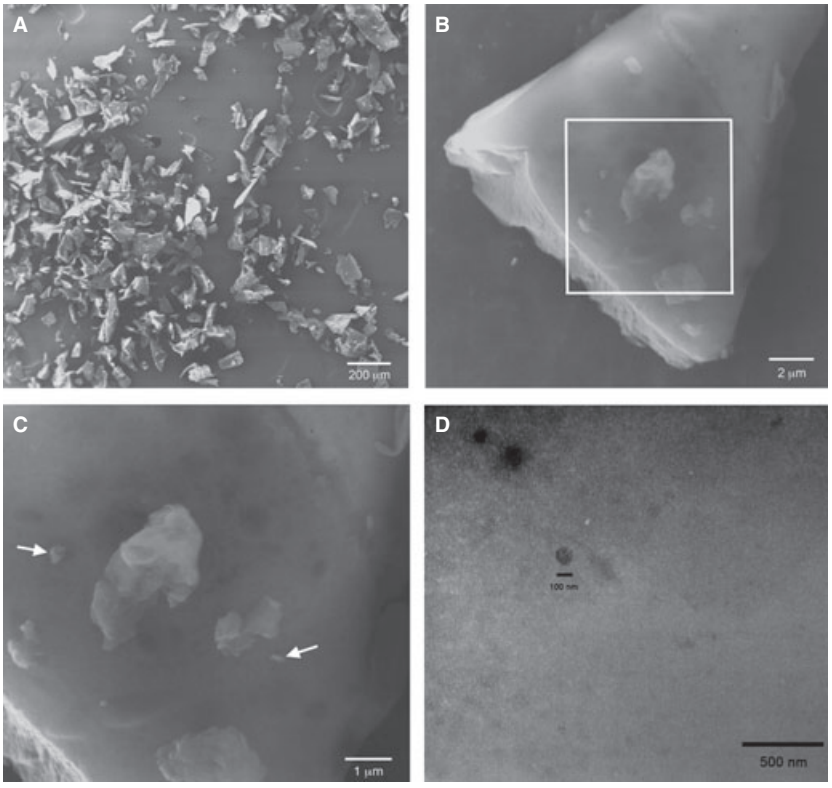

Fig. 1. Morphology of chitosan particles. (A) Low magnification (50×) of a scanning electron microscopy image showing the lyophilized particles under study. (B) Higher-magnification (5000×) image of small particles deposited over a chitosan particle. (C) Inset from (B) showing a detailed view of the smaller particle population (arrows). (D) Transmission electron microscopy image showing the chitosan particles under study.

measurements were not significantly altered in the presence of chitosan, suggesting that this molecule did not induce a cytotoxic effect. Therefore, we selected 100 and 300 µg/mL of chitosan for studying a putative cell-proliferative response induced by this molecule.

Cell viability, measured using the MTS reduction assay, and the total number of cells, detected using crystal violet staining, were then evaluated in serum-starved human gingival fibroblasts, 24, 48 and 72 h after stimulation with 100 and 300 µg/mL of chitosan. Cells were also stimulated with 1 ng/mL of PDGF-BB, a known mitogenic growth factor for gingival cells (18), alone or in combination with chitosan, and with 10% FBS (as a positive control of cell proliferation). As shown in Fig. 3, 100 and 300 µg/mL of chitosan stimulated an increase in cell viability and in the total number of cells detected using crystal violet staining. Cells stimulated with 1 ng/mL of PDGF-BB also showed an increase in cell viability and in crystal violet staining. Interestingly, when PDGF-BB was combined with chitosan particles (100 or 300 µg/mL), we observed, at 48 and 72 h, a statistically significant increase in cell viability and in the total number of cells when compared with 1 ng/mL of PDGF-BB alone (Fig. 3). This response suggested a proliferative effect in gingival fibroblasts induced by chitosan and particularly when chitosan and PDGF-BB were combined.

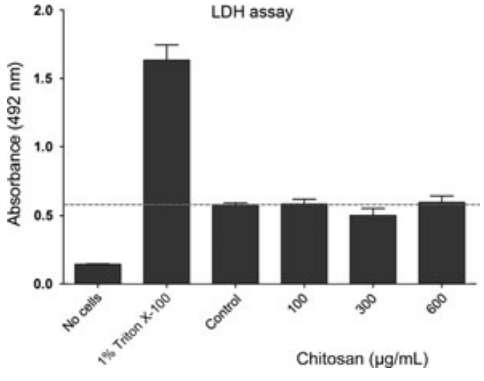

Fig. 2. Lactate dehydrogenase (LDH) assay in gingival fibroblasts exposed to chitosan particles. Gingival fibroblasts were incubated for 48 h in fetal bovine serum-free culture medium containing 100, 300 or 500 µg/mL of chitosan, or no chitosan (Control). One-percent Triton X-100 was used as a positive control of LDH release into the cell-culture medium. Data are presented as mean LDH level + standard error.

Effects of chitosan on PCNA levels

We then evaluated the protein levels of the PCNA, a protein that has previously been used as a marker of cell proliferation (25,26). In order to study a putative synergistic effect between growth factors and chitosan, serum-starved human gingival fibroblasts were stimulated with chitosan (100 or 300 µg/mL) and with FBS at a low concentration (0.5%). In this case, FBS was used as a source of growth factors and proteins that might better reflect an *in-vivo* situation in which these polypeptides are found. As a positive control, cells were stimulated with 10% FBS. As shown in Fig. 4A, the levels of PCNA protein were increased when chitosan and 0.5% FBS were combined. This result suggests a dose-dependent response because 300 µg/mL of chitosan induced a higher PCNA protein level when compared with 100 µg/mL of chitosan. We then evaluated the PCNA protein levels in cells stimulated with 1 ng/mL of PDGF-BB and 300 µg/mL of chito-

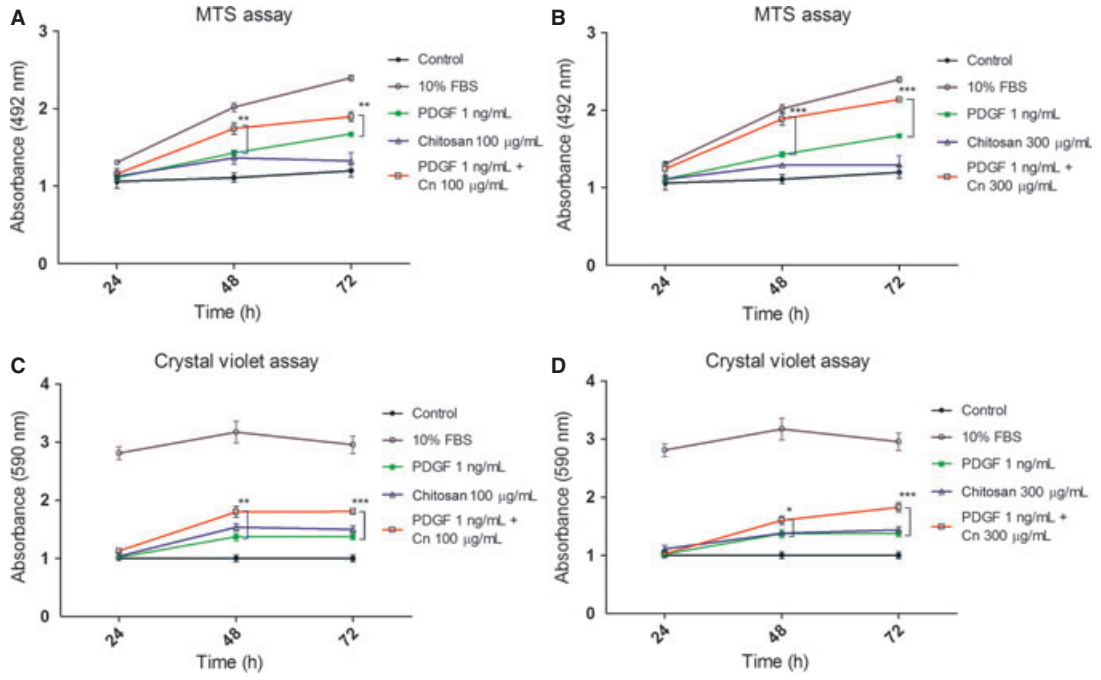

Fig. 3. Effect of chitosan and platelet-derived growth factor (PDGF) on cell viability and cell number. Serum-starved human gingival fibroblasts were exposed to chitosan, PDGF-BB or fetal bovine serum (FBS) for 24, 48 and 72 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (A and B). The number of cells was determined by staining the cells with crystal violet. Stained cells were then solubilized and the absorbance was read using a microplate reader (C and D). Data are presented as mean + standard error of MTS (A and B) or crystal violet (C and D) determinations. Statistical analysis was performed using analysis of variance followed by Scheffé's post-hoc test. *p*-values are shown for the comparisons indicated with brackets. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

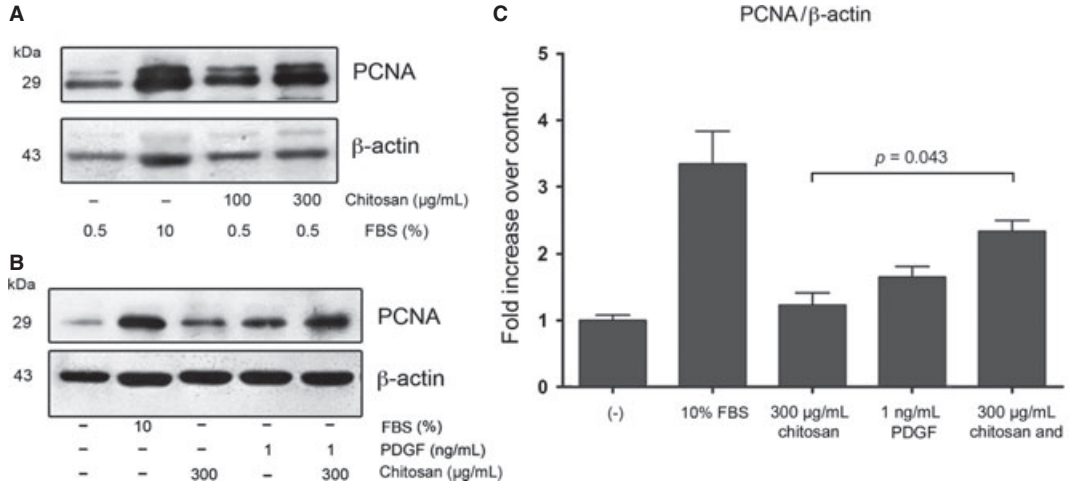

Fig. 4. Effect of chitosan and platelet-derived growth factor (PDGF) on proliferating cell nuclear antigen (PCNA). (A) Human gingival fibroblasts were exposed to chitosan in the presence of 0.5% fetal bovine serum (FBS) for 48 h. (B) Serum-starved human gingival fibroblasts were exposed to chitosan and/or to PDGF for 48 h. In both (A) and (B) the levels of PCNA protein were determined by western blotting of the cell lysates, and β-actin was used as a loading control. (C) Levels of PCNA protein, normalized against β-actin, derived from three independent experiments. Data are presented as mean plus standard error. The bracket indicates a statistically significant difference between the indicated experimental conditions (analysis of variance followed by Scheffé's post-hoc test; the *p*-value is indicated).

san. As shown in Fig. 4B, an increase in the PCNA protein levels was observed when cells were stimulated with 1 ng/mL of PDGF-BB and in particular when PDGF-BB and chitosan were combined. However, chitosan alone did not stimulate the PCNA protein levels when compared with nonstimulated cells.

Quantitative analysis of the results of this experiment showed a statistically significant increase in the PCNA protein levels when PDGF-BB and chitosan were combined compared with cells stimulated with chitosan alone (Fig. 4C).

Effects of chitosan on BrdU incorporation and Ki67 staining

To identify cell proliferation we detected the Ki67 antigen and incorporation of BrdU in cells stimulated with 300 μg/mL of chitosan, 1 ng/mL PDGF-BB or a combination of both agents. As a positive control, cells were stimulated with 10% FBS. The BrdU assay showed an increase in cell staining when cells were stimulated with 300 μg/mL of chitosan, 1 ng/mL PDGF-BB and a combination of both agents (Fig. 5A). Both 100 and 300 μg/mL of chitosan and 1 ng/mL of PDGF-BB stimulated an increase in Ki67 staining compared with non-stimulated cells (Fig. 5A and 5B). Moreover, a combined stimulus of 100 or 300 μg/mL of chitosan with 1 ng/mL of PDGF-BB induced a significant increase in Ki67 staining when compared with PDGF-BB-stimulated cells. In order to identify a synergistic effect between chitosan and the components found in serum, cells were stimulated with 100 or 300 μg/mL of chitosan and 0.5% FBS. In both conditions we observed a statistically significant increase in Ki67 staining when chitosan and 0.5% FBS were combined compared with 0.5% FBS alone.

Role of the ERK1/2 signaling pathway in chitosan-induced cell proliferation

We studied whether chitosan was able to stimulate the activation of ERK1/2, a signaling protein that is involved in cell proliferation (27). To this end,

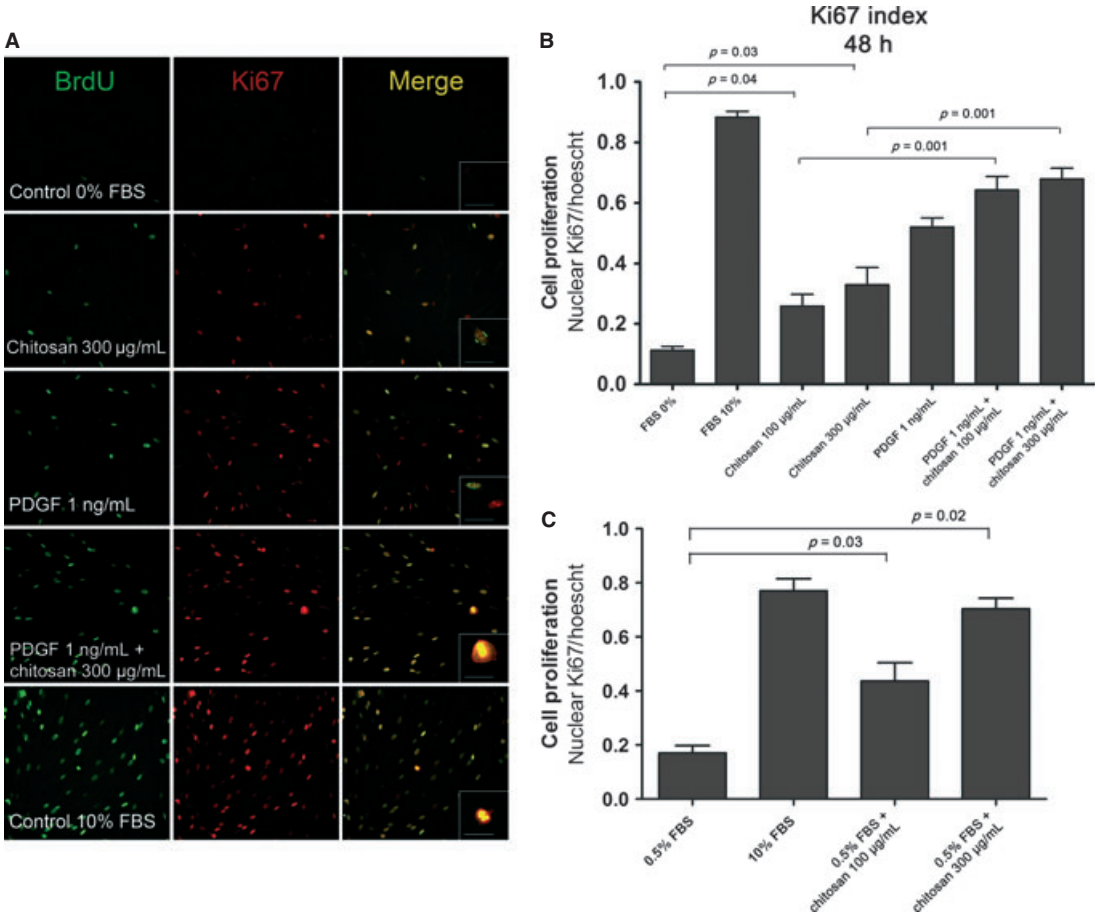

Fig. 5. Effect of chitosan and platelet-derived growth factor (PDGF) on 5′-bromo-2′-deoxyuridine (BrdU) incorporation and Ki67 staining in gingival fibroblasts. (A) Serum-starved human gingival fibroblasts were exposed to chitosan and/or PDGF for 48 h in the presence of BrdU. BrdU incorporation into replicating DNA, and Ki67 staining, were determined using immunofluorescence. The insets depict BrdU- and Ki67-positive nuclei. The positive control was 10% fetal bovine serum (FBS). Scale bar = 10 μm. (B) Numbers of Ki67-positive cells normalized against Hoescht-stained nuclei. Data are presented as mean plus standard error. Brackets indicate statistically significant differences between the indicated experimental conditions (analysis of variance followed by Scheffé's post-hoc test; the *p*-values are indicated). (C) Human gingival fibroblasts were cultured in α-Minimum Essential Medium supplemented with 0.5% FBS and exposed to 100 or 300 μg/mL of chitosan for 48 h. Ki67 and nuclei were stained using immunofluorescence. Data are presented as mean plus standard error of Ki67-positive cells normalized against Hoescht-stained nuclei. Brackets indicate statistically significant differences between the indicated experimental conditions (analysis of variance followed by Scheffé's post-hoc test; the *p*-values are indicated).

serum-starved human gingival fibroblasts were stimulated with 300 μg/mL of chitosan or 1 ng/mL of PDGF-BB. As shown in Fig. 6A, both chitosan and PDGF-BB induced ERK1/2 phosphorylation. It was interesting to note that ERK phosphorylation by chitosan was an early response when compared with ERK activation by PDGF-BB (Fig. 6A and 6B). After analyzing the temporal sequence of ERK activation by both chitosan and PDGF-BB, we selected a 45-min stimulus as an appropriate time-point to identify ERK1/2 activation by both factors. We then stimulated cells with chitosan, PDGF-BB or a combination of both factors in order to evaluate a putative synergistic effect on ERK1/2 phosphorylation. As shown in Fig. 6C and 6D, ERK1/2 phosphorylation was stimulated with chitosan and PDGF-BB, alone, and in combination. However, induction of ERK1/2 phosphorylation by both factors was not statistically different from ERK1/2 phosphorylation induced by PDGF or chitosan alone.

To identify the involvement of the ERK1/2 pathway in the response of gingival fibroblasts, serum-starved human gingival fibroblasts were stimulated with PDGF or chitosan in the presence or absence of a MEK1 inhibitor (PD98059). FBS was used as a positive control. Cell viability was evaluated after 48 h using the MTS assay. As shown in Fig. 7A, PDGF, chitosan and the combination of both factors significantly stimulated cell viability. In all these conditions, the addition of PD98059 significantly inhibited cell viability. To identify the role of ERK1/2 in cell proliferation, the levels of PCNA protein were evaluated after 48 h in cells stimulated with PDGF and chitosan (alone or with PD98059), PDGF + chitosan or PDGF-BB + chitosan + PD98059. As shown in Fig. 7B, when PD98059 was combined with PDGF-BB, chitosan or chitosan + PDGF-BB, a reduction in the levels of PCNA protein was observed. This suggests that ERK1/2 is involved in the signal-transduction pathways activated by chitosan that are necessary to induce cell proliferation in gingival fibroblasts.

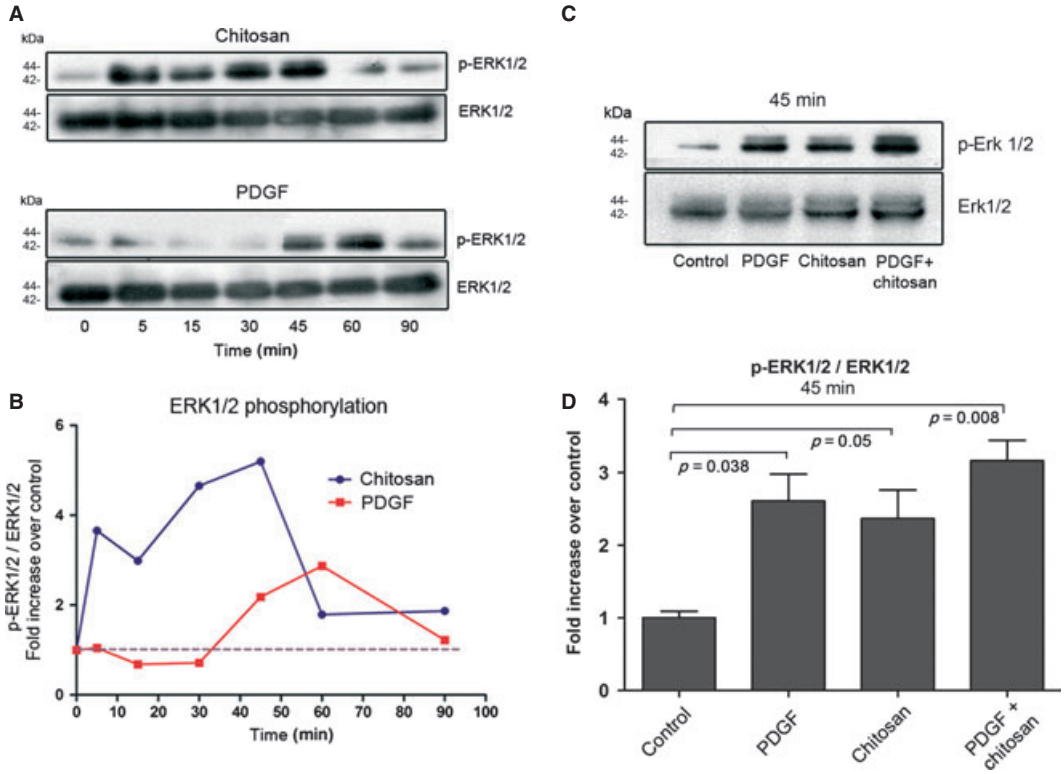

Fig. 6. ERK1/2 involvement in chitosan-stimulated cell proliferation. (A) Serum-starved human gingival fibroblasts were stimulated with 300 μg/mL chitosan or 1 ng/mL PDGF-BB platelet-derived growth factor (PDGF) and phospho-ERK (p-ERK) protein levels were determined by western blotting of the cell lysates. Total ERK levels are shown as a loading control. (B) Time-course graph of mean p-ERK levels, normalized against total ERK levels, after incubation of human gingival fibroblasts with chitosan or PDGF for 0–90min. Dotted line represents baseline p-ERK1/2 values. Data were derived from three independent experiments. (C) Serum-starved human gingival fibroblasts were stimulated for 45 min with chitosan or PDGF, alone, or in combination, and the levels of p-ERK protein were determined by western blotting of the cell lysates. Total ERK levels are shown as a loading control. (D) Bar chart of mean plus standard error p-ERK levels normalized against total ERK levels. Data were obtained from three independent experiments. Brackets indicate statistically significant differences between the indicated experimental conditions (analysis of variance followed by Scheffé's post-hoc test; *p*-values are indicated).

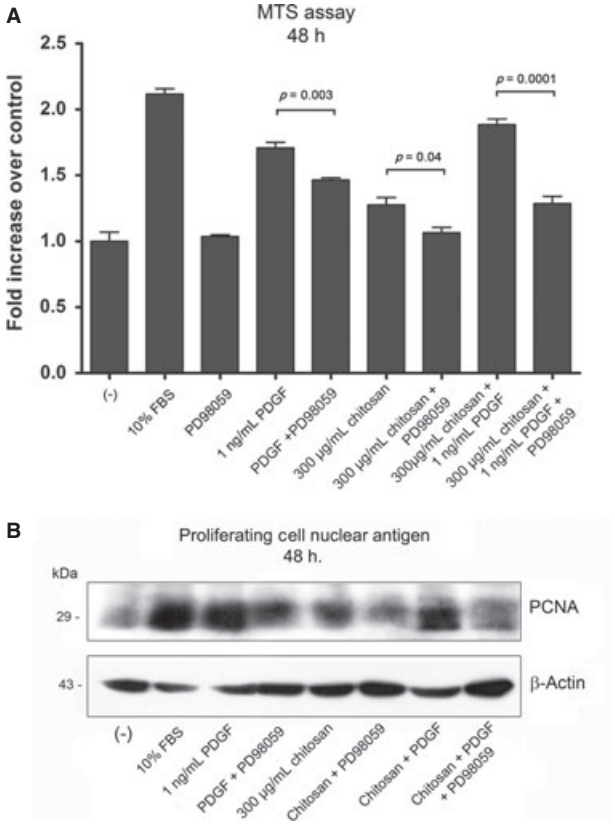

Fig. 7. Modulation of cell viability and proliferating cell nuclear antigen (PCNA) levels by chitosan and growth factors. (A) Serum-starved human gingival fibroblasts were exposed to 300 μg/mL chitosan and/or 1 ng/mL platelet-derived growth factor (PDGF) and/or the MEK1 inhibitor, PD98059, 10 μM for 48 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and the results are presented in the bar chart as mean and standard error. Brackets indicate statistically significant differences between the indicated experimental conditions (analysis of variance followed by Scheffé's post-hoc test; *p*-values are indicated). (B) Serum-starved human gingival fibroblasts were exposed to chitosan and/or PDGF and/or PD98059 for 48 h, and the levels of PCNA protein were determined by western blotting of the cell lysates. The levels of β-actin protein are shown as a loading control.

Discussion

Cell proliferation is a desirable effect of agents used to promote periodontal healing and regeneration. Chitosan has been proposed as a useful biomaterial in periodontal tissue reconstruction because it may promote tissue healing (7) and has antibacterial activity against periodontal pathogens (28–30). In the present study we evaluated the role of chitosan on cell proliferation in primary cultures of human gingival fibroblasts. We observed that chitosan stimulates cell viability (MTS assays) and promotes cell proliferation, as determined through the PCNA protein levels, BrdU incorporation and Ki67 staining. Interestingly, a possible synergistic effect was observed among chitosan, PDGF-BB or other components present in FBS that may stimulate the proliferative response of gingival fibroblasts. Moreover, activation of the ERK1/2 signaling pathway by chitosan may be proposed as an intracellular route that is probably involved in this response. Our results provide evidence of the role of chitosan nanoparticles in promoting cell proliferation in human gingival fibroblasts.

Chitosan has been tested as a gel in association with metronidazole to aid in the treatment of chronic periodontitis and showed a positive effect on tissue healing (10). Another application of chitosan is through the construction of membranes that may facilitate tissue healing by guiding the repopulation of the wound by specific cells (12) or by releasing bioactive molecules, such as growth factors, that may stimulate specific cell responses such as cell proliferation or differentiation (31). In the present study we evaluated the response of gingival fibroblasts to nanoparticles composed of chitosan. We certainly do not know if cell proliferation or other responses induced by chitosan might be different if the chitosan structure is organized as nanostructures or as a macromolecular matrix, as observed in the case of membranes used for tissue regeneration. Future studies should analyze whether the macromolecular organization of the chitosan matrix elicits different responses on gingival or periodontal cells.

In the present study we selected PCNA, BrdU and Ki67 assays to evaluate cell proliferation following stimulation with chitosan. The PCNA is an auxiliary protein of DNA polymerase and it seems to be essential for DNA synthesis (25). More specifically, PCNA can be used as a marker to determine the transition of a cell from the quiescent, or resting, phase (G0 phase) into the DNA-synthesis phase (S phase). Ki67 is a protein expressed in the nucleus during the cell cycle (32). Cells express the Ki67 protein during G1, S, G2 and M phases, but not during G0 (33). BrdU incorporation into replicating DNA has also been used as a test to evaluate cell proliferation (26). As all our experiments gave positive results for the PCNA, Ki67 and BrdU assays, our results are strongly suggestive of the positive effect of chitosan on cell proliferation.

Using Schwann cells, He *et al.* (34) showed that carboxymethylated chitosan was able to stimulate cell proliferation through the activation of ERK and the Akt pathways. The MEK1 inhibitor used in this study has been validated as an appropriate tool to selectively interrupt MEK–ERK signaling (35). We also showed that

chitosan was able to stimulate ERK phosphorylation. However, the mechanisms underlying ERK activation by chitosan remain unknown. Using a polyelectrolyte complex derived from sulfated chitin as a polyanion and chitosan as a polycation, Matsuda *et al*. (36) showed that this macromolecule was able to stimulate ERK1/2 phosphorylation in a human embryonic fibroblastic cell line (HE49). In the same study it was observed that polyelectrolyte complexes did not activate JNK or p38 pathways that are associated with the induction of cellular stress (36). Moreover, ERK1/2 activation was dependent on the activity of focal adhesion kinase, a nonreceptor tyrosine kinase that is activated by integrins (36). There is evidence showing that integrins transduce their signals through focal adhesion kinase phosphorylation, leading to ERK activation (37,38). Although hypothetical, it is possible that integrins might be activated by chitosan particles eliciting the activation of the ERK pathway. Alternatively, other signaling pathways involved in cell adhesion, such as phosphoinositide 3-kinase, cyclic AMP and RhoA, might also be involved in the responses stimulated by chitosan (39).

During wound healing and in response to various extracellular stimuli, cellular commitment to proliferation is regulated by the concerted action of growth factor receptors and integrins that recognize specific extracellular molecules present in the wound environment (40). These responses lead to the activation of diverse signaling pathways and to the induction of several genes involved in cell proliferation (19). Among these signaling routes, ERK activation plays a prominent role in the induction of cell proliferation (41). In skin fibroblasts, PDGF present in the wound fluid may stimulate cell proliferation through the activation of ERK and Akt pathways (42). Moreover, the stimulation of fibroblasts with serum has been used as a model to mimic the response of cells to growth factors present during wound healing (19). In the present study we analyzed the response of fibroblasts to PDGF-BB, which has been identified in gingival wound fluid (43), and also to the wide array of molecules present in serum. It was interesting to note that chitosan induced a synergistic response when this stimulus was combined with PDGF-BB or serum. It is also of interest that cell proliferation stimulated by PDGF in skin fibroblasts is highly dependent on the interaction between the PDGF receptor and integrins (44). Future studies should evaluate whether integrins are involved in the response of gingival fibroblasts to chitosan and growth factors such as PDGF-BB.

In the present study we provide evidence of the proliferative response induced by chitosan in primary cultures of human gingival fibroblasts. This response was dependent on the activation of the ERK1/2 signaling pathway. Moreover, a synergistic response between fibroblasts and growth factors, such as PDGF-BB, was observed. These results are important for understanding the interactions between gingival cells and biomaterials used for the reconstruction of damaged oral tissues.

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