



Enhanced Attachment and Growth of Periodontal Cells on Glycine-Arginine-Glycine-Aspartic Modified Chitosan Membranes

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Background: Chitosan, a polymeric carbohydrate derived from the exoskeleton of arthropod, has been suggested to be an excellent biomaterial for improving wound healing, especially for bones. To improve the periodontal cell attachment and growth, the cell adhesive peptide glycine-arginine-glycine-aspartic acid (Gly-Arg-Gly-Asp, GRGD) grafted chitosan membrane was introduced in this study. **Materials and Methods:** Two types of commercial chitosan, three types of primary cultured cells, and two established cell lines were used. Human gingival and periodontal fibroblasts (hGF and hPDL), human root derived cell (hRDC), and rat calvaria bone cell (rCalB) were cultured on the GRGD-fixed by ultraviolet light photochemical method on the chitosan membrane. With (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay and propidium iodine (PI) staining, the cell adhesion and growth on GRGD-grafted chitosan were examined. Basal mRNA expressions of the receptors for GRGD, integrin α v (ITG α v) and ITG β 3, in the human gingival fibroblast cell line and mouse osteoblast cell line (MC3T3-E1) were examined with real-time polymerase chain reaction. **Results:** Because the cell adhesion/growth patterns on two chitosan membranes were similar, the GRGD modification was performed on one membrane (Primex) only. For periodontal cells (hGFs, hPDLs, and hRDCs), the number of attached cells were increased on the membrane with the high concentration of GRGD than those on the membrane unmodified or modified with low concentration GRGD. For rCalBs cells, a different pattern was noted: GRGD modification did not enhance the calvaria cells attachment or growth. Moreover, mRNA expressions of ITG α v and β 3 in AG09319 cells were significantly higher than those in MC3T3-E1 cells. **Conclusions:** With the limitation of this study, we suggested that GRGD-modified chitosan, especially at high concentration, could enhance the growth of various periodontal fibroblasts, but did not change those of osteoblasts. Therefore, chitosan might be an excellent biomaterial for periodontal use.

Key words: Chitosan, arginine-glycine-aspartic, adhesion, periodontal

INTRODUCTION

The periodontitis is an inflammatory disease in periodontal tissue involving gingiva, cementum, periodontal ligament, and alveolar bone. Eventually, periodontitis will cause tooth loss.¹ To treat the periodontitis, several regenerative procedures, such as guided tissue regeneration (GTR) and tissue engineering, have been widely performed.² However, the details about healing and regenerative mechanisms are still under investigation.³ GTR, for instance, is a common

periodontal regenerative procedure, but still mainly depends on the regenerative ability of periodontium itself.³ One of the major factors for causing the failure of GTR is believed to be the exposure of implanted membrane due to poor adhesion or infection.⁴

Chitosan, a polymeric complex carbohydrate derived from a key constituent of the arthropod exoskeleton, has been suggested to be an excellent biomaterial because of its nontoxic and biocompatible nature.⁵⁻⁷ It has been demonstrated

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that chitosan can improve the proliferation, the differentiation and the mineralization of osteoprogenitor cells, osteoblasts as well as new bone formation.⁸⁻¹⁰ Chitosan has been applied on the surgical procedures of periodontitis, apicoectomy, tooth extraction, and palatal wounds.¹¹⁻¹³ However, studies have shown that chitosan is good for the adhesion and growth of osteoblasts but not of fibroblasts.^{14,15} Recently, the cell adhesive peptide glycine-arginine-glycine-aspartic (Gly-Arg-Gly-ASP GRGD) has grafted to the chitosan membranes to regulate the biological processes of cells because the peptides can be used to probe the cellular integrins.^{16,17} The integrins are transmembrane receptors composed of two subunits α and β that facilitate the anchorage of cells to components of the extracellular matrix or bind to receptors on other cells to support cell-cell adhesion.^{18,19} Integrin binding to extracellular ligands triggers interactions between several signaling molecules in close vicinity to the extracellular and cytoplasmic regions of the integrin chains.^{20,21} Thus, integrins constitute a bidirectional signaling machinery in the membrane that regulates processes such as cell survival, migration, morphology, proliferation, and differentiation.^{20,22} In this study, we applied the GRGD grafted chitosan to examine whether it can improve the adhesion and growth of periodontal cells, especially for those fibroblasts from gingiva and periodontal ligament.

MATERIALS AND METHODS

The preparation of chitosan membrane

In this study, chitosan was purchased from two commercial products: Primex (Ingredients ASA, Norway; batch no. Tm732, Mw of 200–700 kDa with 96% of deacetylation) and Sigma ingredients (Germany, batch no. 44887-7, medium molecular weight with 75–95% deacetylation). To prepare the chitosan solution, 1.0 g of chitosan dissolved in 100 ml of 1% acetic acid solution and then filtered through polycarbonate membrane with the pore size of 0.45 μ m (MS[®] Syringe filter). The solution was poured into glass disks and then solidified in frozen state at -80°C for 7 days to form a thin chitosan film. After the film detached from the glass surface, it was neutralized with 1N sodium hydroxide solution, washed with distilled water until pH was neutral, and dried with hot air.

The grafting of glycine-arginine-glycine-aspartic onto chitosan membrane

GRGD and N-Succinimidyl-6-(4'-axido-2'-nitrophenylamino)-hexanoate (SANPAH), with molecular weight of 403.4 g and 492.4 g, respectively, were purchased from Pierce Chemical Corp. (Rockford, IL, USA). The

procedures to graft GRGD-SANPAH on the surface of chitosan were similar to our earlier study.²³ In general, 0.025M of GRGD and SANPAH were firstly dissolved in distilled water and pure ethanol, respectively. Then, equivalent moles of above-mentioned GRGD and SANPAH solutions were gently mixed and reacted in a dark room at room temperature for 2 h to form phenyl azide-derivatized peptides. The ethanol containing GRGD-SANPAH solution was poured into the above-mentioned chitosan membrane. There were two GRGD concentrations (0.075 and 0.375 mm/cm²) which were made by adding 130 μ l of GRGD-SANPAH and 650 μ l of GRGD-SANPAH solution in 1 cm diameter chitosan membrane, respectively. After the membrane was air dried, they were irradiated by ultraviolet (UV) light (290–370 nm) for 4 min to induce photochemical fixation of GRGD on the chitosan surfaces by a UV generator (Model 68805, ORIEL Instrument, Stratford, CT, USA). The grafting efficiency of GRGD-SANPAH to chitosan was about 83%.¹⁷ The membrane was fully rinsed with distilled water to removed unreacted reagents and then dried at room temperature. The GRGD-grafted chitosan membrane was cut, sterilized with 70% alcohol, and dipped in n-2-hydroxyl-ethylpiperazine-n'-2-ethane sulfonic acid (HEPES) buffer for further sterilization with UV light for 2 days. After the membrane was further rinsed with sterilized HEPES buffer, it was placed on the bottom of a 48 well polystyrene tissue culture plate covered with a sterilized glass ring, 0.8 cm in diameter, to prevent floating.

The preparation of periodontal cells and bone cells

In this study, the primary cultured cells and the established cell lines were used. There were four types of primary cultured cells selected and used in this experiment. They were human gingival fibroblast (hGF), human periodontal fibroblast (hPDL), human root derived cell (hRDC), and rat calvaria bone cells (rCalB). The hGFs were obtained from a gingival spaceman of distal wedge of a 40-year-old female who received periodontal surgery at Tri-Service General Hospital, Taipei, Taiwan. After the specimen was immersed in Leibovitz's L-15 medium (Sigma-Aldrich Inc., St. Louis, MO, USA) with 2 mg/mL Dispase II (Roche Diagnostics, Indianapolis, IN, USA) and 10% fetal bovine serum (FBS) at 4°C for two days, the epithelial layer was separated from the underlying connective tissue. The connective layer was minced into small pieces and then digested in the medium containing 10% FBS and 2 mg/mL collagenase (Sigma-Aldrich Inc.) at 37°C in 5% CO_2 for 24 h to allow the cells to migrate from the explants. The fibroblast cultures were maintained in 10% FBS in Dulbecco's minimum essential

medium (DMEM)/F-12, and incubated until a monolayer was formed.

The hPDLs and hRDCs were derived from an extracted wisdom tooth in the clinics as a medical waste. Briefly, the extracted tooth was rinsed with solution D, phosphate-buffered saline with 1X penicillin/streptomycin (Sigma–Aldrich Inc.), several times. The crown of the tooth was removed, and the pulp canals were enlarged and debrided. The tooth was soaked in 0.2% collagenase with 0.15% trypsin for 2 h. Then, the supernatants were centrifuged in 1500 rpm for 5 min. The same procedures were repeated for three times. All collected cells were pooled as the hPDLs, and cultured in DMEM with 10% fetal calf serum, penicillin, streptomycin, and fungizone for 1 week, whereas the tooth was placed in flask and cells slowly migrated from the root surfaces for 3 weeks were considered as hRDCs according to that described in the previous study.²⁴

In this study, the calvariae were obtained from neonatal male Sprague-Dawley rats which were handled in accordance with the protocols approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, Taipei, Taiwan (IACUC-05-193). After the calvariae were digested in ethylenediaminetetraacetic acid and collagenase, the obtained cells which were mainly primary rCalBs were cultured in 10% FBS DMEM (included 1% antibiotic penicillin/streptomycin). In this study, the first goal was to compare the adhesion and growth of each cell type (hGF, hPDL, hRDC, and rCalB) on the two commercially available membranes. Forty-eight chitosan membranes were used (six membranes for each cell type and company). In each well, 2.5×10^4 cells (including hGF, hPDL, hRDC, and rCalB) with a final cell concentration of 5×10^4 /ml was added where a $0.8 \text{ cm} \times 0.8 \text{ cm}$ chitosan membranes had been preplaced and then grew for 2 days. The adhesion and growth of each cell type were determined.

Quantitative real-time polymerase chain reaction

To examine the basal mRNA expression of GRGD binding receptor integrin α_v (ITG α_v) and integrin β_3 in cells, the purchased human gingival fibroblast cell line, AG09319 (Coriell Institute for Medical Research, Camden, NJ, USA) and the MT3T3-E1 osteoblast cell line (ATCC® CRL-2593™, American Type Culture Collection, Manassas, VA, USA) were further selected and used.

Total RNA was extracted and genomic DNA was eliminated with illustra RNeasy Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). About 1 μg of DNase I-treated total RNA was reverse-transcribed with RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies Corporation, Carlsbad, California, USA) into cDNA and used as the template for real-time polymerase chain reaction (PCR) and analysis. The real-time

PCR reactions were performed using QuantiNova SYBR Green PCR Kit (QIAGEN) on CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The process involved in an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 5 s, and combined annealing/extension at 60°C for 10 s, as described in the manufacturer's instructions. The primer sequences were shown as following: *Homo sapiens* ITG α_v , forward 5'-TCCGATTCCAACTGGGAGC-3' and reverse 5'-AAGGCCACTGAAGATGGAGC-3' (Accession No.: NC_000002.12); *H. sapiens* ITG β_3 , forward 5'-CTGGTGTTCCTACTGATGCCAAG-3' and reverse 5'-TGTTGAGGCAGGTGGCATTGAAGG-3' (Accession No.: NM_000212.2); *H. sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-TGCCAAATATGATGACATCAAGAA-3' and reverse 5'-GGAGTGGGTGTCGCTGTTG-3' (Accession No.: NM_002046); *Mus musculus* ITG α_v , forward 5'-CAGAAAACCAAACCTCGGCAGG-3' and reverse 5'-AGCGAGCAGTTGAGTTCCAG-3' (Accession No.: NM_008402.3); *M. musculus* ITG β_3 , forward 5'-GACAACTCTGGGCGCTC-3' and reverse 5'-GTGGTACAGATGTTGGACTCTCC-3' (Accession No.: NM_016780.2); *M. musculus* GAPDH, forward 5'-CATGGCCTTCCGTGTTCCCTA-3' and reverse 5'-ACTTGGCAGGTTTCTCCAGG-3' (Accession No.: GU214026.1). Calculations of gene expression (normalized to GAPDH reference gene) were performed according to the $2^{-\Delta\Delta C_T}$ method. Fidelity of the PCR reaction was determined by melting temperature analysis.

The evaluation of cell adhesion and growth

The final goal was to evaluate whether the cell adhesion and growth could be enhanced by the modification of GRGD. The polyethylene culture plates were divided into four different groups: (1) Without any membrane; (2) with membranes but without GRGD (the control groups); (3 and 4) two GRGD-modified chitosan membranes with 0.075 and 0.375 mm/cm² in concentrations (the experiment groups). Six samples were included in each group. As previous description, the cells were added to adhere and grow for 2 days and then determined with the cell viability determination assay and PI staining.

The cell viability determination assay was performed measuring the thiazolyl blue [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (the MTS assay) (CellTiter 96_Aqueous One Solution; Promega, Madison, WI, USA) according to the manufacturer's protocol. In this study, the morphology of cells growing on the membrane was further evaluated by

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the PI staining. After washing and fixation, 10 μ g/ml PI was added and reacted with the cells for 30 min. Then, the membranes were elicited with green fluorescent light at 536 nm, observed under red light at 620 nm, and taken the pictures by charge-coupled device camera. All the experiments related to the cell attachment and growth on the chitosan, and the GRGD-modified chitosan membranes had been completed from 2004 to 2007, whereas those experiments related to the cellular expression of GRGD binding integrin of cells were performed recently in 2016.

Statistical analysis

In this preliminary experiment, one-way ANOVA, with the *post hoc* analyzed with a Duncan's test, was selected and used to compare the cell growth of hGF and hPDL, hRDC, and rCalB on the chitosan films. Values were considered to be significantly different only when $P < 0.05$.

RESULTS

Growth pattern of periodontal cells on chitosan membranes

On the two different chitosan membranes, the adhesion and growth patterns of the periodontal cells of hGFs, hPDLs, and hRDCs, as well as the bone cells of rCalBs, were statistically similar [Figure 1]. However, significantly more bone cells (rCalBs) were attached and grown on the membranes if compared with the periodontal cells of hGFs, hPDLs, and hRDCs, regardless of the type of the membrane.

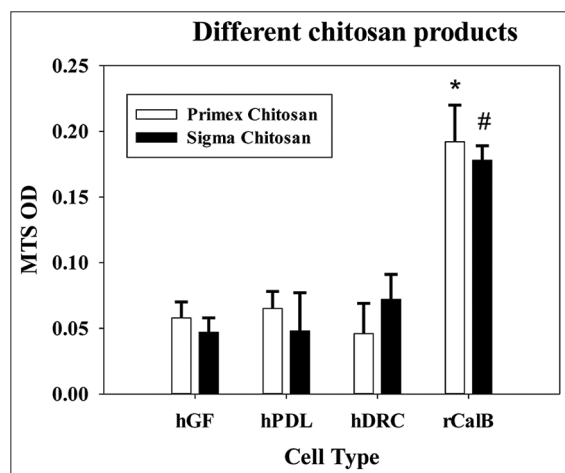


Figure 1: Comparisons of the adhesion and growth of four primarily cultured cells on the two commercially available chitosan membranes (the experiments were repeat three times; means and standard deviations, *significantly different to any other cells, $P < 0.05$). hGF = Human gingival fibroblasts; hPDL = Human periodontal ligament fibroblasts; hRDC = Human root derived cells; rCalB = Rat calvaria bone cells

Growth patterns of periodontal cells on glycine-arginine-glycine-aspartic grafted chitosan membranes

On the Primex membrane modified with the high concentration of GRGD, the number of cells attached for hGFs or hPDLs were significantly higher than that of unmodified or modified with low concentration GRGD [Figures 2a, b, and 3a]. However, it was polyethylene culture plate had the highest number of cells attaching to it. Similar findings were found for hRDCs [Figures 2c and 3c].

A different pattern was noted for the cell attachment of rCalBs [Figure 2d]. The GRGD modification did not enhance the calvaria cells attachment or growth. Furthermore, the number of cell attachment on the polyethylene culture plates was significantly lower.

The different mRNA expression of glycine-arginine-glycine-aspartic receptor in gingival fibroblasts and osteoblasts

The basal mRNA expressions of two different GRGD receptors, ITG α v and integrin β 3, were evaluated in human gingival fibroblast cell line (AG09319) and mouse osteoblast cell line (MC3T3-E1). The mRNA expressions of integrins α v and β 3 in AG09319 cells were significantly higher than those in MC3T3-E1 cells [Figure 4].

DISCUSSION

Recently, the osteoinductive ability of chitosan-collagen composites has been proposed because *in vivo* induced new bone was noted around the titanium implant surface of the subcutaneous tissue of rats in our laboratory.²⁵ An excellent periodontal regeneration in the one-wall alveolar bony defect after the implantation of chitosan has also been observed in an experiment of dog.²⁶ The *in vitro* study has also shown that chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts.¹⁵ In the present study, it could be easily seen in Figure 1 that significantly greater rCalB cell vitality was observed on the chitosan membranes if compared with the cell vitality of periodontal cells (hGFs, hPDLs, and hRDCs), regardless of the membrane types selected. Our previous studies also showed that chitosan could stimulate the activation of platelets and release the growth factors from the platelets.^{27,28} Its ability of enhancing blood clot formation has been proposed based on the findings of a better neoendothelial healing and a thin clot layer completely covering the implanted chitosan-impregnated vascular prostheses.^{29,30} However, the high thrombogenic property would limit the applications of chitosan in blood contacting biomaterials.^{31,32}

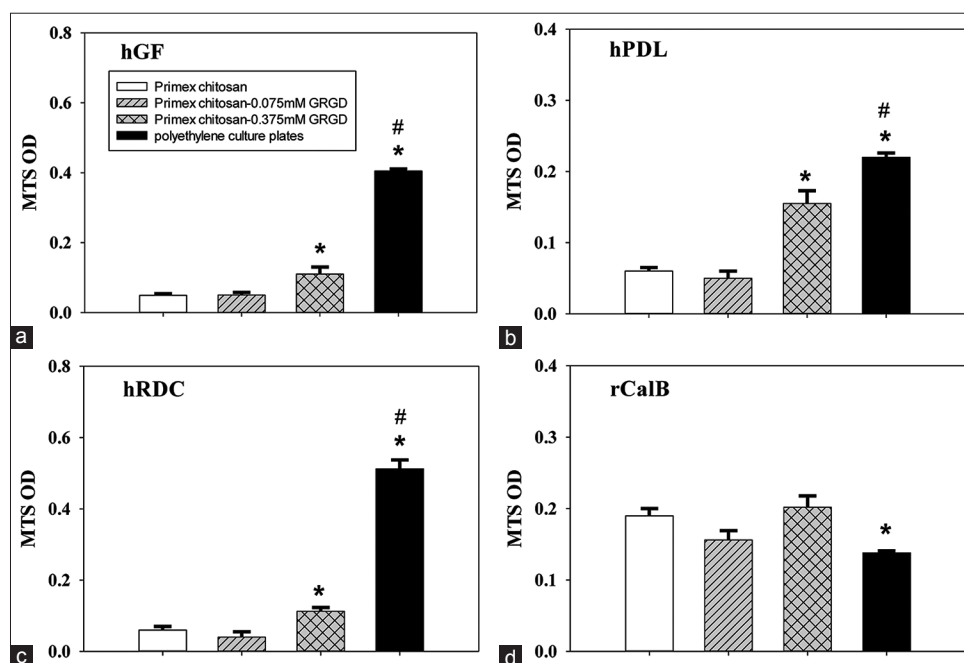


Figure 2: The cell growth of human gingival fibroblasts (a), human periodontal ligament fibroblasts (b), human root derived cells (c), and rat calvaria bone cells (d) in the Primex chitosan membranes was examined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay. (The experiments were repeat three times; means and standard deviations, *significantly different to the cell growth in un-coated Primex membrane, #significantly different to the chitosan membrane, $P < 0.05$)

To improve their blood compatibility, *in vitro* endothelialization using polyurethane on grafted surfaces to provide a bioactive and biological-graft interface has shown promising results in animals.³³ On modified polyurethane surface, many studies have been done,^{33,34} such as photochemically grafted RGD. Photochemical techniques based on phenyl azide chemistry have been applied to graft heparin or RGD peptide to different biomaterial surfaces.^{23,34,35} In those applications, heparin or RGD-peptides were firstly attached to water-soluble functional moiety to form phenyl azido-derivatized polymers or proteins, and then grafted to the material substrates by UV irradiation. Recently, a similar technique to graft GRGD peptide on the surface of chitosan film has been improved by inducing photochemical reactions between azido group and hydroxyl group of the chitosan structure, and then the adhesion and growth of endothelial cells (ECs) on the surface.¹⁷ However, whether it could promote the attachment and growth of cells from the periodontium on the modified chitosan surfaces has still uncertain. The present results presented that the adhesion and proliferation of periodontal cells, including hGFs, hPDLs, and hRDCs, on the chitosan-GRGD surfaces (high concentration group) are more pronounced than that of the original plain chitosan [Figure 2]. However, chitosan-GRGD surfaces does not have the same promoting effect to the bone cells obtained from rat calvarias [Figure 2d]. The cells growth on chitosan

and chitosan-GRGD films are shown after stained with their nuclei [Figure 3]. High concentration of 0.375 mm/cm² GRGD modified chitosan membrane did not increase the 2-day growth, whereas the low concentration GRGD (0.075 mm/cm²) membrane retarded the cell growth.

In general, the adhered cells on those GRGD grafted films are more spread out and dense, whereas those on ungrafted ones are sparser. Since spreading is an essential step in cell adhesion before exponential growth phase,³⁶ a greater extent of cell spreading can have a profound effect on cell growth. Since MTT or MTS assay can reflect the level of cell metabolism,³⁷ the viability for the growth rate of ECs determined by the assay with measuring the absorbance of the formazan solution at 570 nm has been widely applied.^{23,38} Here, the results for MTS assay for viability of growth of periodontal cells on the chitosan and GRGD grafted chitosan films were demonstrated [Figure 2]. Even though polystyrene culture well showed the highest relative growth rate, both GRGD grafted surfaces did enhance the rates by more than 50% when compared with that of ungrafted surfaces. It is well known that RGD tripeptide is the minimal cell recognizable sequence for many adhesion plasma and extracellular proteins including von Willebrand factor, fibronectin, fibrinogen, and collagen.³⁹ In addition, the RGD tripeptide plays a crucial role in mediating cell attachment and subsequently spreading.^{39,40} The enhanced growth rate of periodontal

Cell adhesion on GRGD grafted chitosan membrane

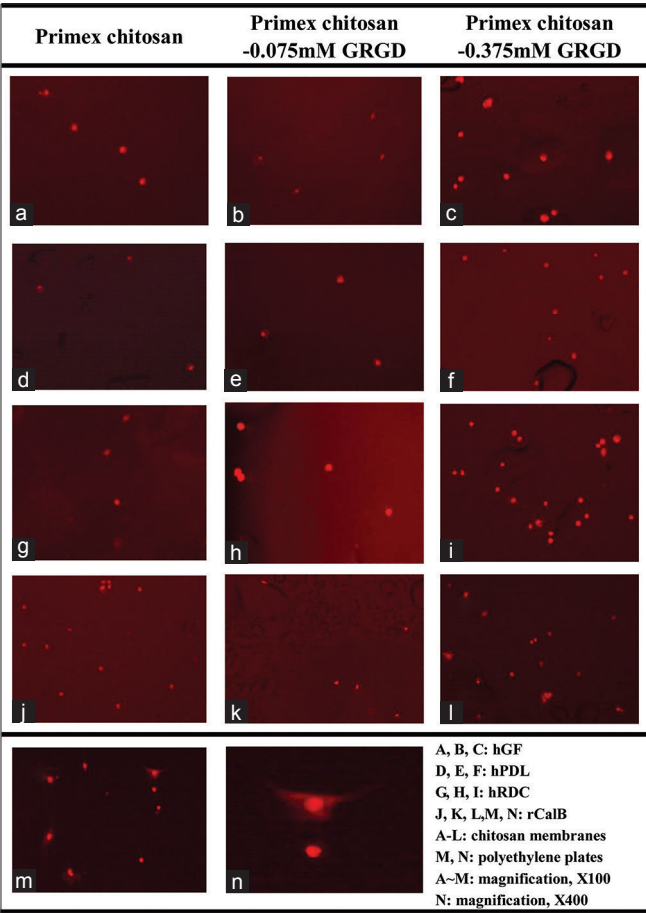


Figure 3: By florescent microscopy, the photographs presenting the cells attached and grown on the ungrafted, grafted with low and high concentrations of glycine-arginine-glycine-aspartic on the primex chitosan membranes (propidium iodine staining, $\times 200$). (a-c) Human gingival fibroblast, (d-f) human periodontal fibroblast, (g-i) human root derived cell, (j-n) rat calvaria bone cell (a-l) chitosan membranes, (m and n) polyethylene plates, (a-m) $\times 100$ (n) $\times 400$

cells on GRGD grafted chitosan surfaces in this study was similar to grafting peptides on polyurethane backbone or polyethylene glycol surface by different methods.^{23,33,35,40} In the present study, the GRGD grafted chitosan membrane was tested to examine whether it could improve the adhesion and growth of periodontal cells, especially for those fibroblasts from gingiva and periodontal ligament. Although the detailed mechanisms for the improvement are still unknown, the basal mRNA expressions of ITG αv and ITG $\beta 3$ GRGD receptors in the gingival fibroblast were significantly higher than those in osteoblast [Figure 4]. It might indicate that the abundant expressions of adhesion molecules in the fibroblast might play an important role for the cell attachment. In fact, scalloped chitosan applied in tissue engineering for culturing hepatocytes, fibroblasts, and cartilage cells has been reported.⁴¹⁻⁴³ The chitosan has also been used as the carrier for delivery of drugs

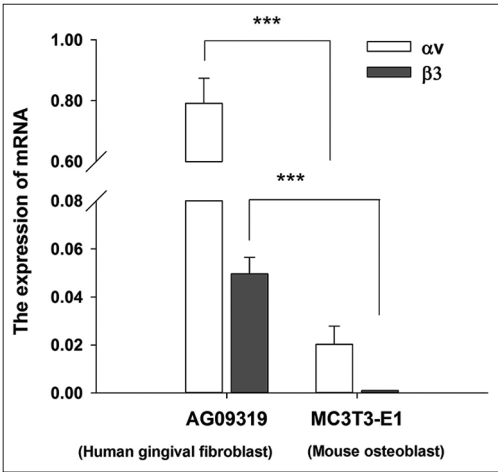


Figure 4: The mRNA expressions of glycine-arginine-glycine-aspartic binding receptor integrin αv and integrin $\beta 3$ in gingival fibroblast (AG09319) and osteoblast (MC3T3-E1) (the experiments were repeat three times; means and standard deviations, ***significantly different to AG09319, $P < 0.001$)

or growth factors to promote wound healing.⁴⁴ Moreover, the method of RGD modification has been recently applied for the adhesion and spreading and differentiation of varied types of cells including the stem cells.⁴⁵⁻⁴⁹ With the limitation of the present study, we suggested that GRGD, especially at high concentration, modified chitosan surface could significantly enhance the growth of varied of periodontal fibroblasts but may not change those of osteoblasts. The GRGD modified chitosan might be an excellent biomaterial for periodontal use.

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Conflicts of interest

There are no conflicts of interest.

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