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Degradation of collagen-guided tissue regeneration membranes by proteolytic enzymes of *Porphyromonas gingivalis* and its inhibition by antibacterial agents

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Abstract
Previous studies have shown that whole cells of several periodontal pathogenic bacteria including *Porphyromonas gingivalis* may degrade the clinically used regeneration membranes Biomend Extend™ and Bio-Gide®. Fractionation of *P. gingivalis* cells revealed that cell membrane-associated proteases are responsible for the *in vitro* degradation of the collagen membranes. In the present study, the specific role of extracellular vesicles and the purified Arg-gingipain enzyme of *P. gingivalis* in the degradation of three differently cross-linked collagen membranes (Ossix™; Bio-Gide® and Biomend Extend™) was examined. In addition, the inhibitory effect of antibacterial agents and antibiotics used in local periodontal therapy on the enzymatic degradation was evaluated. The data presented show that while all tested collagen membranes, are prone to lysis by oral bacterial proteases, cross-linked membranes are more resistant to proteolysis. Furthermore, therapeutical concentrations of the antibacterial and antibiotic agents chlorhexidine, cetylpyridiniumchloride, minocycline and doxycycline were found to partially inhibit the enzymatic breakdown of the membranes, while metronidazole had no such effect. These results suggest that the presence of *P. gingivalis* cells, extracellular vesicles and enzymes in the vicinity of regeneration membranes in the periodontium, may change their physical structure and therefore alter their biological properties. Furthermore, the use of cross-linked collagen membranes and antibacterial agents may significantly inhibit this proteolytic process.

The use of guided tissue regeneration (GTR) and guided bone regeneration (GBR) for the treatment of periodontal defects, or in conjunction with dental implant procedures is widespread (Bunyaratavej & Wang 2001; Taguchi et al. 2005; Strietzel et al. 2006). GTR have become a routine treatment method for management of bone defects resulting from periodontal diseases not responding to conservative therapy.

Several studies have suggested that optimal tissue regeneration cannot be expected for barrier membranes placed in sites infected by periodontopathic microorganisms (Nowzari & Slots 1995; Nowzari et al. 1995; De Sanctis et al. 1996a, 1996b; Chen et al. 1997; Hämmerle & Karring 1998; Smith MacDonald et al. 1998; Yoshinari et al. 1998; Cortellini & Tonetti 2000; Sela et al. 2003).

A prerequisite for treatment success is therefore an infection-free healing process. Rudiger et al. (2003) studied the dynamics of bacterial colonization in intra-osseous defects following GTR therapy. They have suggested that the colonization of periodontal pathogens at sites treated by GTR included *Actinobacillus* (*Aggregatibacter*)

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actinomycetemcomitans, *Porphyromonas gingivalis*, and *Bacteroides forsythus* (*Tannerella forsythensis*) and could be correlated with the intra-oral presence of these pathogens before surgery. Bacterial adhesion and colonization of exposed GTR membranes used in conjunction with implants, may cause the development of infection in the underlying treated tissues and result in partial regeneration or implant failure (Jovanovic et al. 1992; Mellonig & Triplett 1993; Landsberg et al. 1994; Simion et al. 1994a). Several periodontal pathogens were shown to be responsible for unsuccessful regeneration procedures.

Mombelli et al. (1993) found that Gram-negative, anaerobic rods made up 31% of all organisms cultivated from e-PTFE membranes. Nowzari et al. (1995) showed that *P. gingivalis* could be detected in sites with loss of probing attachment after e-PTFE membrane removal. They also demonstrated that *Prevotella intermedia*, *Peptostreptococcus micros*, and *Campylobacter rectus* constituted high proportions of the bacteria found on membranes in sites demonstrating little or no attachment. Furthermore, Selvig et al. (1992) and Machtei et al. (1994) suggested that the amount of bacteria found on regeneration membranes may be used as a predictor of the outcome of the regenerative treatment. They suggested that in order to ensure successful regeneration, periodontal pathogens should be controlled in the site of membrane insertion.

The periodontal pathogenic bacteria: *Treponema denticola* and *P. gingivalis*, were shown to adhere to different barrier membranes *in vitro*, and cause rapid degradation of collagen membranes (Sela et al. 1999, 2003; Sela 2001). Moreover, the examined bacteria demonstrated a significantly high affinity to collagen membranes. The proteolytic activity of periodontal pathogenic bacteria such as *P. gingivalis* is suggested to have a crucial effect on cells and tissues of the periodontium. Gingipains of *P. gingivalis* are responsible for the majority of the proteolysis including the collagenolytic activity of these bacteria (Imamura 2003). Arg-gingipains A and B are specific for – Arg–Xaa and are derived from two genes *rgp*A and *rgp*B, while Lys-gingipain is specific for – Lys–Xaa peptide bonds and is derived from a single gene *kgp* (Potempa et al. 1995, 2000; Curtis et al. 2001).

These enzymes, which are part of the metabolic bacterial cycle, were shown to degrade several human proteins with high biological importance. These include among others: native collagens I, III, IV and V, fibrin, fibrinogen, fibronectin, protease inhibitors and immunoglobulins (Kadowaki et al. 1994; Abe et al. 1998). Degradation of matrix proteins together with the activation of matrix metalloproteinases contributes to the role of *P. gingivalis* in periodontal tissue destruction. Furthermore, these bacterial proteases were suggested to act in the evasion and modulation of the immune system, activation of the coagulation system, enhancement of vascular permeability and fibrinogen degradation which may contribute to the bleeding tendency associated with periodontal diseases (Kadowaki et al. 2000; Imamura 2003).

Since early degradation of collagen membranes is detrimental to the success of regenerative procedures, the inhibitory effect of chemical processes and materials on bacterial adherence and early degradation were examined. Recent studies that have examined modified resorbable GBR–GTR membranes with different antibacterial properties, suggested that membranes loaded with such materials might enhance GTR efficacy. These include among others mineralization with zinc phosphate shown to inhibit oral bacterial colonization (Chou et al. 2007) and tetracycline impregnation which delayed collagen membrane degradation *in vivo* (Zohar et al. 2004; Lee et al. 2008).

The aim of the present study was to examine the ability of *P. gingivalis* purified proteases, Arg-gingipain and extracellular vesicles (representing the bacterial surface proteolytic capability), to degrade bioabsorbable collagen tissue regeneration membranes with or without cross-linkage. Furthermore, several antibacterial agents used locally for the treatment of periodontal disease were examined for their effect on the degradation of collagen membranes by the oral bacterial enzymes.

Materials and methods

Bacterial strains and growth conditions

P. gingivalis – ATCC 33277 was grown in a medium described by Duchesne et al. (1995) supplemented with 0.05% cysteine and 0.4 ml of resazurin solution (0.25 mg/ml). The bacteria were incubated in an anaerobic chamber in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 at 37°C.

Vesicle and HRgpA preparation

Purification of enzymes was performed essentially as described by Rangarajan et al. (1997). Briefly, bacterial cultures were centrifuged (8000 *g*, 60 min, 4°C), and the supernatant was separated from the pelleted cells. The supernatant was treated with ammonium sulfate (85% saturation) and the precipitated protein was centrifuged at 8000 *g*, 60 min at 4°C. The pellet was solubilized in 50 mM acetate buffer (pH 5.3) containing 0.0055% Zwittergent. The insoluble material was separated by centrifugation and designated as extracellular vesicles, or used for further purification of HRgpA. The supernatant (soluble fraction) obtained after separation of the vesicles contains two forms of Arg-gingipain: HRgpA and RgpA. HRgpA was purified from either the soluble fraction or the vesicles by affinity chromatography on a Arginine-sepharose column as described before (Rangarajan et al. 1997). HRgpA purification from the vesicles (that contain HRgpA, RgpA, and *m*-RgpA), was conducted by resuspension in 100 mM sodium acetate buffer and 1% Zwittergent and further centrifugation, the supernatant containing HRgpA was submitted to Arginine-sepharose affinity chromatography. Enzyme purity was examined by gel electrophoresis. The purified enzyme showed an apparent subunit size of 54 kDa on reducing SDS-PAGE.

Enzyme activity assays

Enzymatic activity of Arg-gingipains was measured with the colorimetric substrate *N*-a-benzoyl-L-arginine-*p*-nitro-anilide as described before (Sela et al. 2003). In order to examine the proteolytic activity in the presence of antibacterial agents, azocoll was used as described by Chavira et al. (1984). Determination of collagen membrane degradation was tested by either a fluorescence labeling technique (Sela et al. 2003) or by the release of hydroxyproline as described by Stegemann & Stadler (1967), and modified by Redlich et al. (1994).

Collagen membranes

The following collagen membranes were used in the study: *Bio-Gide*® – Geistlich Biomaterials, Wolhausen, Switzerland (Porcine collagen types I and III). *Biomend Extend*™ – Integra Life Sciences Corporation, Sultzer Calcitek Inc., Carlsbad, CA, USA (compressed, non-friable type I bovine collagen matrix). *Ossix*™– Colbar Ltd., Ramat Hasharon, Israel (type I beef tendon collagen).

Preparation of fluorescent collagen membranes

The membranes were submitted to a fluorescence labeling technique (Sela et al. 2003). They were immersed in a 0.01 M NaOH (pH 9) solution for 10 min, then washed with water, acetone, and further incubated overnight with fluorescein isothiocyanate 0.6 mg/ml in acetone in the dark at 4°C. After labeling, the membranes were exhaustively washed with acetone, water, and TBS until the fluorescence in the washings reached basal levels. The membranes were either used immediately or kept at 4°C in the dark and then washed with acetone and dried. After proteolysis, the supernatant fluid was removed and the fluorescence was measured with excitation and emission wavelengths at 490 and 520 nm, respectively. All fluorescence measurements were made in a PTI spectrofluorometer (Photon Technology Industries Co., South Brunswick, NJ, USA) at 25°C.

Proteolytic degradation of the membranes

The collagen membranes were cut into 5 mm × 5 mm pieces weighed and incubated with either purified HRgpA or vesicles in 0.2 M Tris-HCl (pH 7.6) containing 10 mM $CaCl_2$ and 10 mM cysteine. The reference value, corresponding to 100% lysis of the collagen membranes was obtained by incubating the membranes with collagenase (1 mg) from *Clostridium hystolyticum* (Sigma type V, 125 U/mg solid), in 0.2 M Tris-HCl (pH 7.6) containing 10 mM $CaCl_2$. Collagen membranes incubated in the respective buffer solutions served as controls. Unless otherwise stated, incubations were performed for 16 h at 37°C, under aerobic conditions.

Inhibition of the proteolytic degradation of the membranes

Antibacterial agents used locally for the treatment of periodontal disease were examined for their effect on the Arg-gingipain activity of the *P. gingivalis* vesicles and on their proteolytic degradation of the collagen membranes. These included: cetylpyridiniumchloride (CPC), chlorhexidine, metronidazole, minocycline and doxycycline. Inhibition of the vesicles' Arg-gingipain activity was performed by preincubating the vesicles with the inhibitor for different time intervals. After preincubation, remaining Arg-gingipain activity was measured by the colorimetric method as described above. Alternatively, the vesicles were incubated with the collagen membranes and released hydroxyproline was determined as described above.

Statistics

The ANOVA model with repeated measures was applied in order to test the effects of time, inhibitors, concentrations and the interactions between them. The Greenhouse-Geisser test was used to assess significance for the time effects. The one-sample *t*-test was used in order to test whether results for different inhibitors and concentrations were different from control (where the mean of the remaining activity was different from 1). The non-parametric Kruskal–Wallis test was applied to test the difference between three independent groups or more. The Mann–Whitney non-parametric test and the one-sample *t*-test were used to assess difference between two independent groups.

Results

The capability of *P. gingivalis* purified HRgpA, or extracellular vesicles containing the Arg- and Lys-gingipains of these bacteria, to degrade collagen membranes was evaluated. Clostridium collagenase was used as the reference positive control. Three units of Arg-gingipain activity (the minimal saturating degrading activity) were compared with 75 U of Clostridium collagenase, which degraded the membranes [Bio-Gide® (non-cross-linked), Biomend Extend™ (cross-linked by formaldehyde) and Ossix™ (cross-linked by D-ribose)] completely. As shown in Fig. 1, *P. gingivalis*-purified HRgpA was able to degrade 80% of the non-cross-linked Bio-Gide® membrane while extracellular vesicles degraded 90% of this membrane. The cross-linked membranes Biomend Extend™ and Ossix™ were significantly less degraded by either the purified HRgpA or the extracellular vesicles ($P<0.01$) (Fig. 1). On the other hand, the differences between the degradation of the two cross-linked membranes, either by the vesicles or the purified enzyme were not significant ($P>0.05$). These data suggest that Arg-gingipains are the main *P. gingivalis* vesicles proteases responsible for collagen membranes degradation.

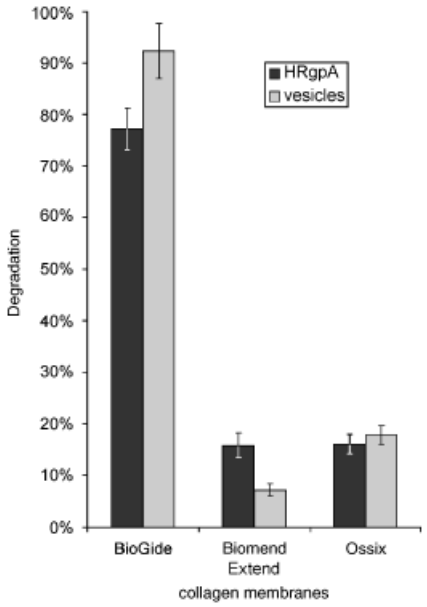

Fig. 1. Degradation of the collagen membranes Bio-Gide, Biomend Extend™ and Ossix™ by *Porphyromonas gingivalis*-purified HRgpA or extracellular vesicles. Collagen membranes were incubated with HRgpA (3 U) or extracellular vesicles (3 U of Arg-gingipain activity) for 16 h at 37°C. Degradation (%) was determined by soluble fluorescence measurement or hydroxyproline release as described in 'Materials and methods.'

The effect of antimicrobial agents on the proteolytic degradation of Bio-Gide® collagen membranes, which was highly degraded by *P. gingivalis* proteases, was further studied. Chlorhexidine and CPC (0.05% and 0.2%) significantly inhibited the vesicle proteolytic activity in a concentration ($P<0.001$) and preincubation time ($P<0.05$) dependent manner (Fig. 2). Preincubation of the vesicles (180 min) with 0.05% or 0.2% chlorhexidine inhibited the Arg-gingipain activity by 22% and 39%, respectively, and preincubation with CPC (0.05% or 0.2%) showed a stronger Arg-gingipain inhibitory activity; 36% and

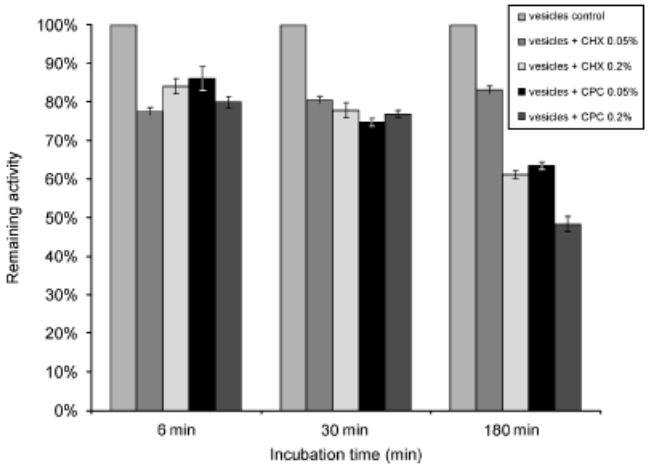

Fig. 2. Time and concentration dependent inhibition of *Porphyromonas gingivalis* extracellular vesicle Arg-gingipain activity by chlorhexidine or cetylpyridiniumchloride (CPC). Vesicles were preincubated with chlorhexidine or CPC at the stated times and inhibitor concentrations, before determination of Arg-gingipain activity.

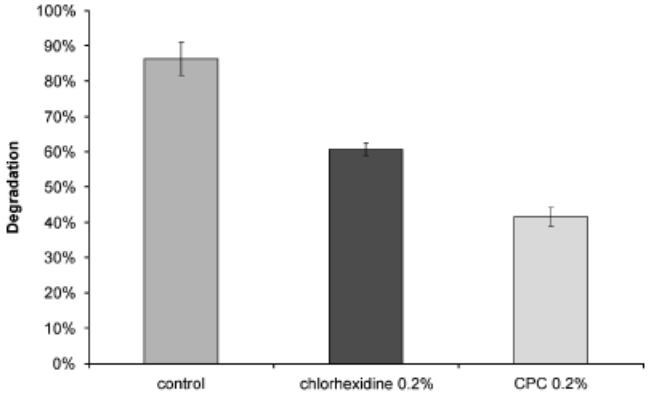

Fig. 3. Inhibition of collagen membrane degradation by *Porphyromonas gingivalis* extracellular vesicles after preincubation with chlorhexidine or CPC. Vesicles (3 U of Arg-gingipain activity) were preincubated with either chlorhexidine (0.2%) or CPC (0.2%) for 30 min previous to their incubation with the collagen membranes for 16 h at 37°C.

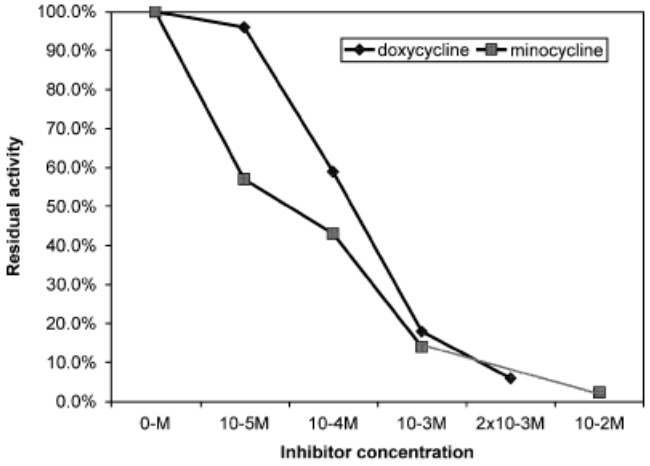

Fig. 4. Concentration dependent inhibition of *Porphyromonas gingivalis* extracellular vesicle Arg-gingipain activity by doxycycline and minocycline. Vesicles were preincubated with the antibiotic agents at the stated inhibitor concentrations before determination of Arg-gingipain activity.

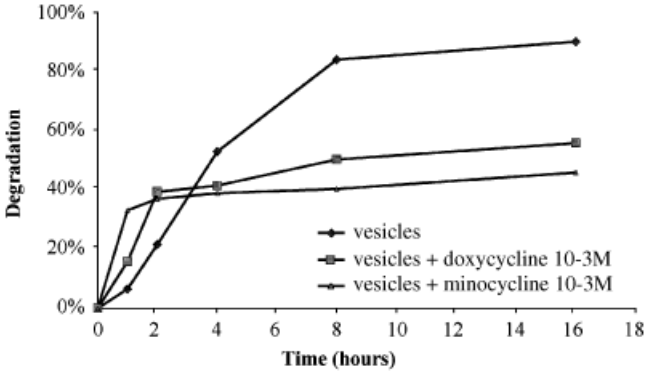

Fig. 5. Inhibition of collagen membrane degradation by *Porphyromonas gingivalis* extracellular vesicles after preincubation with doxycycline or minocycline. Vesicles (3 U of Arg-gingipain activity) were preincubated with either antibiotics (10^{-3} M), for 30 min previous to their incubation with the collagen membranes for 16 h at 37°C.

52%, respectively. Under the same conditions metronidazole had no inhibitory effect on the Arg-gingipain activity (data not shown). Preincubation of the vesicles with 0.2% chlorhexidine reduced the degradation of the collagen membranes by the bacterial enzymes by 25%, while preincubation with 0.2% CPC resulted in an inhibition of 44% of the enzymatic degradation (Fig. 3). When the membranes were impregnated with either of the inhibitors (20% chlorhexidine or 10% CPC), before incubation with the *P. gingivalis* vesicles, no significant reduction in proteolytic degradation was observed (data not shown). Furthermore, preincubation of *P. gingivalis* vesicles with either 10^{-3} M of minocycline or doxycycline, inhibited the proteolytic activity by 90% (Fig. 4), and resulted in the inhibition of the collagen membranes degradation by 36% and 44%, respectively (Fig. 5).

Discussion

We aimed to evaluate the role of purified *P. gingivalis* proteases in biodegradation of clinically used collagen barrier membranes constructed by different cross-linking techniques and to test the effect of clinically used antimicrobial agents on *P. gingivalis*-mediated degradation of these membranes. The data presented demonstrate that cross-linked collagen membranes (Biomend Extend™ and Ossix™) are significantly less degraded by *P. gingivalis* proteases than non-cross-linked membranes (Bio-Gide®). In addition, clinically used concentrations of CPC, chlorhexidine, minocycline, and doxycycline significantly inhibited the *P. gingivalis* mediated-enzymatic degradation of these membranes.

Being one of the major causative factors of periodontal diseases, *P. gingivalis* produces large amounts of arginine- and lysine-specific cysteine proteinases in cell-associated and secretory forms, referred to as Arg-gingipain (Rgp) and Lys-gingipain (Kgp), respectively. Both Rgp and Kgp

were shown to be able to degrade extracellular matrix proteins including collagens I, III, IV and V, fibronectin and laminin. These asaccharolytic bacteria, utilize Rgp and Kgp to obtain nutrients, as carbon and energy sources for growth. They are therefore totally dependent on peptides and amino acids that are provided from environmental proteins by Rgp and Kgp.

Furthermore, three different *P. gingivalis* peptidases capable of hydrolyzing peptide bonds containing proline residues have been described. These include a prolyl tripeptidyl peptidase (Banbula et al. 1999) and a prolyl dipeptidyl peptidase (Banbula et al. 2000). These peptidases have the potential to hydrolyze collagen-derived peptides which are rich in proline residues generated by collagenases.

The presence of enzymes like Rgp, Kgp, and the prolyl peptidases in *P. gingivalis* together with their ability to adhere to collagen membranes (Sela et al. 1999) may explain the role of these oral bacteria in the degradation of GTR collagen membranes.

Bacterial enzymatic degradation of GTR membranes in the periodontium is of importance in light of the fact that bacterial infection may lead to the failure of GTR processes. Only few *in vivo* studies examined the outcome of regeneration in the presence of bacteria. Mombelli et al. (1993) demonstrated that e-PTPE membranes used for periodontal regenerative procedures were frequently colonized by periodontal bacteria. Wang et al. (1994) who studied the early adhesion of a variety of bacteria to three GTR membranes *in vitro*, demonstrated the occurrence of selective adherence patterns of bacteria to different regeneration membranes. Further studies showed a direct correlation between the presence of bacteria on e-PTFE and polyglycolactic membranes and reduction in the potential gain of probing attachment level (De Sanctis et al. 1996a, 1996b). A correlation between the presence of *A. actinomycetemcomitans* and the regenerative outcome *in vivo* was demonstrated by Machtei et al. (1994). In a comparison between adhesion to barrier membranes surrounding teeth and implants, Nowzari & Slots (1995) could associate putative periodontal pathogens with GBR failure. Bacterial passage through membranes accompanied by fibroblasts and giant cells exposure to the oral cavity was also demonstrated (Simion et al. 1994a, 1994b; Leghissa & Botticelli 1996). These clinical and *in vitro* findings suggest that the composition and/or structure of the membrane may play an important role in the clinical outcomes of bacterial infection.

Our findings that bacterial proteases degrade collagen membranes together with the ability of *P. gingivalis* to adhere to the membranes, may explain, in part, the deleterious role of bacterial infection in the failure of GTR treatment. The activity of bacteria and their enzymes may bring to the rapid elimination of membrane material and shorten the desired period of regeneration considerably. In this study, we tested two possible approaches to extend the biodegradation of collagen membranes used for GTR. Namely the use of cross-linked collagen membranes and the addition of antibacterial agents utilized locally in periodontal therapy. Our results suggest that the cross-linking structure of collagen membranes is associated with prolonged biodegradation by *P. gingivalis* proteases. It could be speculated that the specific chemical composition of the cross-linked membranes (e.g. the presence of covalently modified amino acids) makes them more resistant to proteolytic degradation by the bacterial enzymes. It is interesting to note that Tal et al. (2008) who evaluated long-term barrier bio-durability of cross-linked and non-cross-linked collagen membranes in sites treated by GTR procedures have found that cross-linked membranes were more resistant to tissue degradation.

Previous studies that examined the effect of antibacterial agents such as chlorhexidine, tetracycline, minocycline and doxycycline on gingipains, revealed that these antibacterial agents may act as effective proteolytic inhibitors independent of their antimicrobial activity. (Imamura et al. 2001; Grenier et al. 2003; Cronan et al. 2006). Furthermore, *in vivo* studies (Zohar et al. 2004) have demonstrated that soaking collagen membranes in 50 mg/ml tetracycline hydrochloride solution may act as a useful and practical tool to slow membrane degradation.

We evaluated the effect of the antibiotics: minocycline, doxycycline, and metronidazole as well as the antimicrobial agents: CPC and chlorhexidine for their inhibitory effect on collagen membrane degradation by *P. gingivalis* proteases.

Preincubation of *P. gingivalis* vesicles with chlorhexidine, CPC, minocycline, and doxycycline inhibited the proteolytic activity of the Arg-gingipains in solution as well as their ability to degrade the collagen membranes, metronidazole on the other hand, had no inhibitory effect. While, inhibition of gingipains by chlorhexidine and tetracyclines has been reported in the past, inhibition of *P. gingivalis* enzymes by CPC, is demonstrated for the first time. This finding might have a clinical significance because CPC which is a cationic quaternary ammonium compound is widely used as an antimicrobial agent in mouthwashes and toothpastes.

The differences found in enzymatic inhibition between chlorhexidine, CPC, and metronidazole may be due to differences in their molecular charge or the ability of these antibacterial materials to change the enzymatic conformation.

Furthermore, the inhibitory activity of chlorhexidine and CPC may be due to their effect on the protease itself, rather than on the collagen barrier membrane. The cationic (positively charged) nature of these agents makes them unlikely candidates to bind with, or have a high affinity for basic (positively charged) amino acids such as arginine on the membranes' surface. It is more likely that they have a high affinity for portions of the active site of the protease, which subsequently bind to the arginine substrate.

Our results may point to the need of a synchronized handling of the tissues during the GTR process by membranes and antibacterial agents which act as protease inhibitors. This approach may prevent alterations in tissue collagenase activity and may act more efficiently than the impregnation of inhibitors into the membranes before their insertion and use *in vivo*.

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