Reduction of Proteolytic Degradation by Chlorhexidine
D. Grenier

J DENT RES 1993 72: 630
DOI: 10.1177/00220345930720031301

The online version of this article can be found at:
http://jdr.sagepub.com/content/72/3/630

Published by:
http://www.sagepublications.com

On behalf of:
International and American Associations for Dental Research

Additional services and information for Journal of Dental Research can be found at:

Email Alerts: http://jdr.sagepub.com/cgi/alerts

Subscriptions: http://jdr.sagepub.com/subscriptions

Reprints: http://www.sagepub.com/journalsReprints.nav

Permissions: http://www.sagepub.com/journalsPermissions.nav

Citations: http://jdr.sagepub.com/content/72/3/630.refs.html

>> Version of Record - Mar 1, 1993

What is This?
Reduction of Proteolytic Degradation by Chlorhexidine

D. GRENIER

Groupe de Recherche en Ecologie Buccale, Faculté de Médecine Dentaire, Université Laval, Ste-Foy, Québec, Canada G1K 7P4

The present investigation was designed to evaluate the effect of the antimicrobial agent chlorhexidine on proteolytic degradation. Chlorhexidine, at a final concentration of 0.01%, significantly (p = 0.05) affected the degradation of the general chromogenic substrate azocoll by both commercial proteases and cell-bound bacterial proteases. Reduction of proteolytic degradation was observed following pre-treatment of either azocoll or bacterial cells with chlorhexidine. Chlorhexidine was also found to prevent degradation of type I collagen by Porphyromonas gingivalis cells. The mechanism of inhibition of proteolytic degradation appears to be related to an electrostatic chlorhexidine-protein interaction.


Introduction.

Inflammatory periodontal diseases are characterized by a significant breakdown of connective tissues (Page and Schroeder, 1973). A number of proteolytic enzymes have been found in periodontal sites [reviewed by Sandholz (1986)]. For example, Kowashi et al. (1979) reported an increase in collagenase and neutral protease activities in the gingival sulcus during experimental gingivitis in man. In addition, a positive correlation has been demonstrated between trypsin-like activity in subgingival sites and both the level of clinical disease and the populations of Porphyromonas gingivalis and spirochetes (Sueldo et al., 1985).

Chlorhexidine is a cationic bisbiguanide which exerts its antimicrobial effect by binding to extra-microbial complexes and negatively-charged microbial cell envelopes, resulting in a disruption of cytoplasmic membrane functions (Hugo and Longworth, 1964). This antimicrobial agent possesses a broad spectrum of activity against oral bacteria, including suspected periodontopathogens such as Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans (Oosterwaal et al., 1989; Stanley et al., 1989). Over the last decade, the use of chlorhexidine as an adjunct to periodontal therapy has been the topic of several studies. Efficient control of supragingival plaque and a reduction in gingival inflammation by chlorhexidine mouthrinses have been confirmed by different groups (Löe and Schiött, 1970; Flötra et al., 1972; Lang and Breex, 1986; Flemmig et al., 1990). A number of studies have also indicated that subgingival irrigation with chlorhexidine can reduce periodontal inflammation, sulcular bleeding, pocket depth, and subgingival plaque (Soh et al., 1982; Wieder et al., 1983; Jolkovsky et al., 1990). Recently, it has been demonstrated that chlorhexidine reduces plaque accumulation as a result of both immediate bactericidal action during application as well as prolonged bacteriostatic action due to its adsorption to the pellicle coating the enamel surface (Jenkins et al., 1988). The aim of the present investigation was to evaluate the effect of chlorhexidine on proteolytic degradation by both commercial proteases and cell-bound bacterial proteases.

Materials and methods.

Proteolytic enzymes and bacteria.—The commercial enzymes (Boehringer Mannheim Canada, Laval, QC) used in the present study were bovine pancreatic trypsin (10 μg/mL), bovine pancreatic chymotrypsin (100 μg/mL), and proteinase K (10 μg/mL). Solutions were made in distilled water and kept at -20°C for up to one month. P. gingivalis ATCC 33277 was grown (16 h) in brain-heart infusion broth (BBL Microbiology Systems, Cockeysville, MD) containing hemin (10 μg/mL) and vitamin K (1 μg/mL). Treponema denticola ATCC 35405 was cultivated (5 d) in the NOS medium, as previously described (Leischine and Canale-Parola, 1980). Cultures were incubated in an anaerobic chamber (N2, H2, CO2, 80-10-10) at 37°C. The bacterial cells were harvested by centrifugation at 10,000 g for 15 min and suspended in distilled water to an A600 of 1.0 for P. gingivalis and 2.0 for T. denticola.

Effect of chlorhexidine on azocoll-degrading activity.—Chlorhexidine diacetate (Sigma Chemical Co., St. Louis, MO) was prepared in distilled water at the following concentrations (w/v): 0.2%, 0.05%, and 0.0125%. The inhibitory effect of chlorhexidine was measured by monitoring hydrolysis of the general chromogenic substrate azocoll (Calbiochem, La Jolla, CA) by proteolytic enzymes. Briefly, chlorhexidine solution or distilled water (100 μL) was incubated in the presence of either commercial enzymes or bacteria (100 μL), 50 mmol/L potassium phosphate buffer, pH 7.2 (300 μL), and 1.5 mg of azocoll. When bacterial suspensions were used, 5 mmol/L dithiothreitol and 0.2 mol/L sodium chloride were included in the phosphate buffer so that bacterial protease activity would be stimulated. The mixtures were then incubated at 37°C for 2 h (16 h in the case of T. denticola cells). The optical density at 520 nm was recorded spectrophotometrically following centrifugation (10,000 g for 5 min) of the assay mixture. The inhibitory effect of chlorhexidine was expressed as % activity of the control assay (no chlorhexidine). Alexidine, another bisbiguanide compound, was also tested for its ability to reduce proteolytic degradation.

Nature of the inhibition.—The nature of the inhibition was assessed by pre-incubation (at room temperature for 2 h) of either the azocoll or the P. gingivalis cells with 0.04% chlorhexidine (final concentration). The pre-incubated azocoll or bacterial cells were then washed four times.
times in phosphate buffer, pH 7.2 (1.5 mL with gentle agitation for 5 min), prior to performing the proteolytic assay. The effect of being washed with 100 mmol/L phosphate buffer adjusted to pH 3.0 was also evaluated.

Effect of chlorhexidine on the collagen-degrading activity of P. gingivalis.—Chlorhexidine diacetate was prepared in distilled water at the following concentrations (w/v): 0.05%, 0.0125%, and 0.003%. The inhibitory effect of chlorhexidine on collagen degradation was determined by assay for the production of lower-molecular-weight fragments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the Laemmli (1970) buffer system. Briefly, P. gingivalis cells (50 μL; A₆₆₀ = 1.0 in distilled water) were incubated in the presence of 100 mmol/L Tris hydrochloride-0.5 mmol/L dithiothreitol buffer, pH 7.0 (100 μL), acid-soluble type I collagen (50 μL; 1 mg per mL of 0.01% acetic acid), and either distilled water (50 μL) or chlorhexidine solution (50 μL). After 4 h at 37°C, the assay mixture was boiled for 10 min in the presence of 2% SDS and 0.5% 2-mercaptoethanol, and run on SDS-PAGE (8% polyacrylamide). The proteins were stained with Coomassie brilliant blue.

Statistical analysis.—All experiments were carried out in triplicate, and the mean ± standard error of the mean (SEM) was calculated. For each assay, the data were analyzed by analysis of variance (ANOVA) with the GLM (General Linear Models) procedure. The significance of the differences between the various groups was analyzed by a multiple-comparison test (Fisher's least-significant difference) with p = 0.05 as the level of significance.

Results.

The effect of chlorhexidine on proteolytic degradation by various proteolytic enzymes is presented in Table 1. Chlorhexidine inhibited the degradation of azocoll by both commercial proteases (trypsin, chymotrypsin, and proteinase K) and cell-bound bacterial proteases (P. gingivalis and T. denticola). All proteolytic activity was significantly reduced (p = 0.05) when chlorhexidine was used at a final concentration of 0.01% and 0.04%. The degradation of azocoll by bovine pancreatic chymotrypsin and the protease activity of P. gingivalis cells were the most affected. In both cases, less than 30% of the activity of the control assay was obtained in the presence of chlorhexidine at a concentration of 0.01%. When alexidine was used, instead of chlorhexidine, no significant reduction in proteolytic degradation was observed. It was also found that the use of a synthetic chromogenic peptide such as N-α-benzoyl-L-arginine-p-nitroanilide (BAPNA) instead of azocoll required a higher amount of chlorhexidine for a similar inhibition of activity to be obtained (data not shown).

Preliminary experiments were carried out to characterize the inhibition mechanism involved. Pre-incubation of azocoll with 0.04% chlorhexidine resulted in a reduction of the proteolytic degradation of the substrate by both bovine pancreatic chymotrypsin and P. gingivalis cells (Table 2). When the chlorhexidine pre-incubated azocoll was extensively washed at pH 3.0 and then at pH 7.2, its susceptibility to proteolytic enzymes was fully restored. A control assay indicated that treatment of azocoll (without pre-incubation with chlorhexidine) at pH 3.0 did not modify its degradation by proteolytic enzymes.

### TABLE 1

<table>
<thead>
<tr>
<th>Pro tease or Bacterial Cells</th>
<th>% Activity of Control&lt;br&gt;(Chlorhexidine Concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin</td>
<td>48 ± 10&lt;sup&gt;2&lt;/sup&gt;&lt;br&gt;0.01&lt;sup&gt;1&lt;/sup&gt; 0.0025&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trypsin</td>
<td>98 ± 11&lt;sup&gt;1&lt;/sup&gt;&lt;br&gt;0.04&lt;sup&gt;1&lt;/sup&gt; 0.01&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>49 ± 9&lt;sup&gt;2&lt;/sup&gt;&lt;br&gt;3 ± 2&lt;sup&gt;2&lt;/sup&gt; 6 ± 1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. gingivalis Cells</td>
<td>69 ± 12&lt;sup&gt;1&lt;/sup&gt;&lt;br&gt;2 ± 1&lt;sup&gt;1&lt;/sup&gt; 2 ± 1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>T. denticola Cells</td>
<td>81 ± 12&lt;sup&gt;1&lt;/sup&gt;&lt;br&gt;45 ± 9&lt;sup&gt;1&lt;/sup&gt; 3 ± 2&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean ± SEM of three separate assays.<br>Significantly different from the control assay (no chlorhexidine) at p = 0.05.

### TABLE 2

| Assay                                | pH of Washing Buffer | % Activity of Control<br>|p| |
|--------------------------------------|----------------------|-------------------------|
| Chymotrypsin + CH<sup>1</sup>-pre-incubated azocoll | 7.0 | 15 ± 4<sup>2</sup> |
| Chymotrypsin + CH-pre-incubated azocoll | 7.0 | 2 ± 1<sup>1</sup> |
| P. gingivalis + CH-pre-incubated azocoll | 7.0 | 37 ± 7<sup>1</sup> |
| P. gingivalis + CH-pre-incubated azocoll | 7.0 | 98 ± 11 |
| CH-pre-incubated P. gingivalis + azocoll | 3.0 | 98 ± 11 |

Mean ± SEM of three separate assays.<br>CH: chlorhexidine at a final concentration of 0.04%.<br>Significantly different from the control assay (no chlorhexidine) at p = 0.05.
the chlorhexidine-treated azocoll at pH 7.2 alone did not restore its susceptibility to chymotrypsin or to P. gingivalis proteases. Similar results were obtained when P. gingivalis cells, instead of azocoll, were pre-incubated with 0.04% chlorhexidine (Table 2).

The ability of chlorhexidine to interfere with the degradation of type I collagen by P. gingivalis cells was determined by SDS-PAGE analysis. Hydrolysis of collagen was completely abolished in the presence of chlorhexidine at a final concentration of 0.01%, and partially prevented at a final concentration of 0.0025%.

Discussion.

Tetracycline, an antibiotic used in the treatment of periodontal disease, has been reported to inhibit, by its chelating properties, the collagenolytic activity of polymorphonuclear leukocytes (Golub et al., 1983). Inhibition of enzymatic processes by chlorhexidine, an antimicrobial agent, has also been reported. Recently, Beighton et al. (1991) demonstrated the ability of chlorhexidine to inhibit a variety of glycosidase and peptidase activities of oral bacteria. The glycosyltransferase from Streptococcus mutans, which may be important in dental plaque formation, was found to be highly sensitive to chlorhexidine (Scheie and Kjellei, 1987). In addition, the phosphoenolpyruvate-phosphotransferase system of this microorganism is also inhibited by chlorhexidine (Marsh et al., 1983). Since proteases are thought to play an important role in inflammatory diseases such as periodontal disease (Harris and Cartwright, 1977), the present study was undertaken to evaluate the effect of chlorhexidine on proteolytic degradation.

Chlorhexidine was found to have a significant effect on the degradation of azocoll by a variety of proteolytic enzymes showing different specificities. It appears that chlorhexidine binds to both the substrate and the bacterial cells, thus preventing the proteolytic process. The interaction is likely to be via electrostatic forces, since the reduction in proteolytic degradation was reversed following treatment at pH 3.0. In vitro reactions of chlorhexidine with proteins have been previously reported (Hjeljord et al., 1973). The binding sites have been suggested to be mainly carboxyl and phosphate groups (Gjerme et al., 1975; Waaler, 1990). The inhibition of the cell-associated proteolytic activities of P. gingivalis and T. denticola, observed in the present study, is unlikely to result from a specific binding of chlorhexidine to the active site of the enzyme. Rather, it is suggested that a general cell envelope modification is responsible for the reduction in proteolytic activity.

Recently, Minhas and Greenman (1989) reported the effect of chlorhexidine on P. gingivalis cells grown in continuous culture. They found that the level of trypsin-like activity decreased significantly when the bacteria were cultivated in the presence of chlorhexidine. According to the results of the present study, this reduction may result from an inhibition of the activity rather than a decreased production of hydrolytic enzyme(s).

In addition to being bactericidal against a variety of bacterial species found in periodontal sites, subgingival application of chlorhexidine may interfere with protein degradation occurring during periodontal diseases. It may also, as suggested by Beighton et al. (1991), affect the growth as well as reduce the virulence of suspected periodontopathogens. The long-term retention of chlorhexidine in the oral cavity (Gjerme, 1975) may provide the above beneficial effects over a long period of time.

Acknowledgments.

I thank Dr. H. Scott (University of British Columbia) for her helpful comments, and G. Bourgeau (Université Laval) for editorial assistance.

REFERENCES


Marsh PD, Keeliv CW, McDermid AS, Williamson MI, Ellwood DC (1983). Inhibition by the antimicrobial agent chlorhexidine of...


