CURRENT MICROBIOLOGY Vol. 43 (2001), pp. 96–99
DOI: 10.1007/s002840010268

Current Microbiology
An International Journal

Identification of Photolabile Outer Membrane Proteins of *Porphyromonas gingivalis*

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Received: 30 August 2000 / Accepted: 1 January 2001

Abstract. As the prevalence of antibiotic-resistant strains of bacteria increases, novel ways of treating infections need to be developed. This is particularly pertinent with respect to the periodontal diseases—the most common chronic bacterial infections of man. The use of a photosensitizer in combination with red light has been demonstrated to be effective in killing several human pathogens, including the oral bacterium, *Porphyromonas gingivalis*, a major pathogen in periodontitis. Killing was associated with alterations in the molecular masses of several outer membrane and plasma membrane proteins and these may be therapeutic targets for photodynamic therapy and other antimicrobial approaches. To identify these photolabile proteins, we have used a panel of monoclonal antibodies raised to whole *P. gingivalis*. A number of the antibodies recognized various photolabile proteins. Using a combination of Western blotting and protein sequencing the predominant photolabile proteins in *P. gingivalis* have been identified as the major secreted/cell surface proteases—Lys and Arg gingipain.

Porphyromonas gingivalis is a Gram-negative, obligately anaerobic, coccobacillus which is believed to be a major pathogen in periodontitis, one of the most common of the chronic infectious diseases of man [1]. The selective killing of this organism would be of enormous benefit in the therapy of this disease, which is estimated to affect up to 15% of the world's population [2]. We have previously reported that *P. gingivalis* can be rapidly killed *in vitro* if it is exposed to the photosensitizer, toluidine blue O, and Helium-Neon laser light [3]. Analysis of the cells after exposure to photosensitizer and light revealed changes in the molecular masses of several outer membrane (OM) and plasma membrane (PM) proteins [4]. Photodynamic killing is not selective for bacteria, but could be made selective, and potentially more effective, if it were targeted to these photolabile proteins, for example, by linking the photosensitizer to a monoclonal antibody recognizing these proteins. In addition, these proteins may be targets for other anti-bacterial modalities.

In this study we have used a panel of monoclonal antibodies (mAbs) raised to *P. gingivalis* to identify the OM and PM proteins which undergo photo-induced alterations.

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Materials and Methods

Laser and photosensitizer. The laser used in this study was a helium/neon (HeNe) gas laser (NEC Corporation, Japan) with a measured output of 35 mW which emits light in a collimated beam (diameter of 1.3 mm), with a wavelength of 632.8 nm. The photosensitizer used was toluidine blue O (TBO, Sigma Ltd., Poole, UK). *P. gingivalis* W50 was maintained by twice-weekly subculture on fastidious anaerobe agar (FAA, Lab M). The bacteria were incubated at 37°C in an anaerobic cabinet (Don Whitley Scientific Ltd, Bradford, UK). For experimental purposes, a few colonies of *P. gingivalis* were inoculated into Bacteroides medium (BM) which consisted of the following tryptone soya broth (10 g), protease peptone (10 g), yeast (5 g), glucose (5 g), sodium chloride (5 g), and cysteine-HCl (0.75 g) per litre of distilled water. The

pH was adjusted to 7.5, and the broth was autoclaved at 121°C for 15 min. The medium was supplemented with hemin (Sigma Ltd., Poole, U.K.) and menadione (Sigma Ltd., Poole, UK) prior to use so that the final concentrations were 5 μg/ml and 0.5 μg/ml, respectively. The culture was incubated in an anaerobic chamber until it reached stationary phase (approximately 24 h).

Buffers and reagents. (A) Photosensitizer: A stock solution of 50 μg/ml TBO was prepared in saline (0.85 %, wt/vol) and filter-sterilized. (B) Protease inhibitor: Bacterial samples were resuspended in 10 μM (final concentration) of Nα-pTosyl-L-Lysinechloro-methyl ketone (TLCK Sigma Ltd., Poole, UK), which was dissolved in distilled water. (C) CAPS buffer: A stock solution (x10) of CAPS (3-[Cyclohexyl-amino]-1-paropanesulphonic acid) was prepared by dissolving 22.13 g of CAPS in 900 ml water (AnalR, BDH) and then titrated with NaOH to a pH of 11.0 and adjusted to a final volume of 1 litre. A working solution was prepared by adding 100 ml of CAPS (x10) to 100 ml of methanol (AnalR, BDH) and 800 ml water (AnalR, BDH). (D) Buffer I: Phosphate buffered saline, pH 7.4 containing 1% Triton X-100. (E) Buffer II: Phosphate buffered saline, pH 7.4 containing 0.1% Triton X-100. (F) Blocking buffer: Buffer II containing 2% skimmed milk powder (Safeways, UK).

Lethal photosensitization of *P. gingivalis*. Stationary phase *P. gingivalis* was harvested and resuspended in saline (0.85 %, wt/vol). The suspension was aliquoted (100 μl) into wells of a 96-well microtitre plate and 100 μl of saline or 50 μg/ml TBO were added. After a pre-irradiation time of 60 s, the suspensions containing TBO were exposed to laser light for 15 min (corresponding to an energy dose of 31.5 J). The samples were continually stirred with microstirring bars (BDH) throughout the duration of the experiment. After this time, the cells were harvested and resuspended in 100 μl of distilled water containing 10 μM of TLCK and 40 μl of sample buffer. The samples were boiled for 4 min and were separated (30 mA for 45 min) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins for sequencing were separated on 10% gels. After electrophoresis, the gels were rinsed in ultrapure water containing 0.2% (wt/vol) dithiothreitol (DTT, Sigma Ltd., Poole, UK) and incubated in CAPS buffer for 10 min with one buffer change after 5 min. After this time, the gels were electroblotted onto PVDF membranes (BioRad, UK) which were pre-soaked for 15 min in 100% methanol and then 1 h in CAPS buffer containing 0.2% DTT. The electroblotting was carried out at room temperature for 1 h at 500 mA using CAPS buffer. After electroblotting, the membranes were placed in buffer I for 5 min and then buffer II for 25 min. After this time, the membranes were transferred to blocking buffer for 45 min. The membranes were then incubated for 2 h with primary antibody in blocking buffer. A range of monoclonal antibodies were used, some of which recognized capsular material, some LPS, while others recognized undefined antigens of *P. gingivalis* [5]. The membranes were washed in buffer II for 30 min with frequent buffer changes and then incubated with secondary antibody (anti-mouse antibody, peroxidase-conjugated, Sigma Ltd, Poole, UK) in blocking buffer for 1 h. The membranes were washed once again in buffer II as described above and then incubated in DAB (3,3′-diaminobenzidinetetrahydrochloride containing 0.01% hydrogen peroxide) until protein bands were visible.

The whole procedure was repeated using antibody 7BD2 as this recognized differences in the protein profiles between TBO/light-treated bacteria and bacteria which were not treated with either. However, in this case, an additional gel was run and prepared for sequencing as described below. The membrane was washed in water containing 2% DTT and was then placed in 0.2% Coomasie blue R-250 in 5:4:1, methanol:water:acetic acid for 3 min. The membrane was destained in the above solvent and then rinsed with water containing 2% DTT. The membrane was soaked in 2% DTT for 10 min and the bands of interest were cut from the membrane using the antibody-probed membrane for reference. Protein bands were subjected to Edman degradation sequencing of the first 15 amino acids (Dr. J. Gray, University of Newcastle). The sequence data obtained were compared with protein and nucleotide sequence databases using the National Centre for Biotechnology Information BLAST network service.

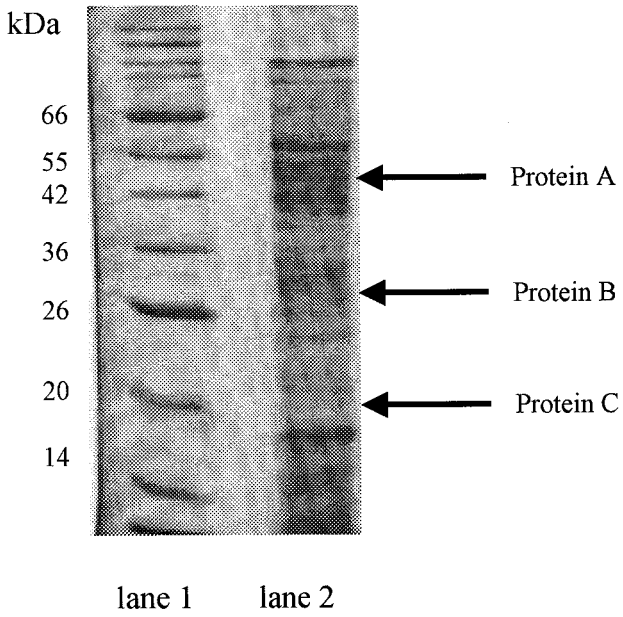

Fig. 1. SDS-PAGE gel of untreated (L-S-) *P. gingivalis* cells stained with 0.2% Coomassie blue. Arrows indicate the protein bands (in lane 2) that were sequenced. Lane 1 shows the molecular mass markers.

Results

In order to select an appropriate antibody for use in detecting differences in the protein profiles of treated and untreated bacteria it was necessary to screen a range (thirteen) of different antibodies recognising different cell surface components of *P. gingivalis*. Most of these revealed that there was a reduction in the intensity of bands corresponding to proteins with molecular masses of 50, 29, and 20 kDa upon treatment with TBO and laser light. This was accompanied, in most cases, by additional bands in the treated cells—these corresponded to proteins with molecular masses of 205 and 66 kDa.

Further work was carried out with one particular antibody, 7BD2, as this gave very clear-cut, repeatable protein patterns. This antibody recognized proteins with molecular masses of 50kDa (protein A), 29kDa (protein B) and 20kDa (protein C). Recognition of proteins A and C by the antibody was reduced following treatment with TBO and laser light. Protein B was no longer recognized by antibody in the treated samples. Figure 1 shows a coomassie blue stained gel of whole *P. gingivalis* cells

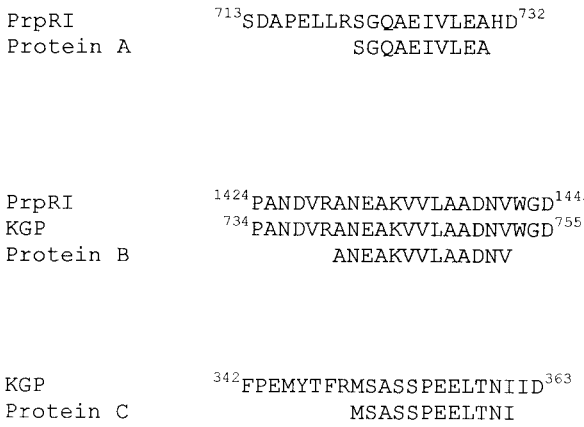

Fig. 2. Alignment of the N-terminal sequences obtained for proteins A (50kDa), B (29kDa) and C (20kDa) with matches from the SWISS-PROT database.

separated by SDS–PAGE indicating the protein bands which were sequenced. Proteins A, B, and C, were sequenced and had the following N-terminal sequences: Protein A: S-G-Q-A-E-I-V-L-E-A; Protein B: A-N-E-A-K-V-V-L-A-A-D-N-V, Protein C: M-S-A-S-S-P-E-E-L-T-D-I-I-D-K.

The N-terminal sequences obtained were compared to sequences on the SWISS–PROT database using the FASTA algorithm to identify the proteins. The sequence data for protein A was identical to amino acids 721–730 of the arginine-specific thiol protease [6] of *P. gingivalis*, PrpRI. The sequence data for protein B revealed identity with regions in two proteins on the database, amino acids 1430-1442 of PrpRI and amino acids 740–753 of the Lys-gingipain protein [7] of *P. gingivalis*, KGP. The sequence data obtained for protein C was identical to amino acids 350–363 of KGP. The alignments of the N-terminal sequences with database matches are shown in figure 2.

Discussion

Our laboratories have previously shown that outer and plasma membrane proteins of *P. gingivalis* are affected by cytotoxic species produced when TBO-treated bacteria are irradiated with red light [4]. We have also previously shown that treatment of *P. gingivalis* with TBO followed by irradiation with laser light decreases the amount of proteolytic activity secreted by this bacterium [8].

In this study we have used a range of antibodies for Western blotting to determine differences in the protein banding patterns between TBO/laser-light treated bacteria and untreated bacteria. Differences were nearly always in the 50, 29, and 20 kDa region (data not shown). Interestingly, many of the Western blots showed proteins appearing at around the 150–200 kDa region in treated samples. This may be due to cross-linking of the proteins induced by the cytotoxic species produced during lethal photosensitization.

Sequence analysis showed that one of the photolabile proteins (A) was a part of PrpRI which has been previously identified by examination of the specific immune responses of individuals with a history of destructive periodontal disease [6]. PrpRI has two forms: monomeric (ArgIA consisting of the α subunit) and dimeric (ArgI consisting of the α and β subunits). The complete gene (*prpRI*) has the potential to encode for a 175 kDa protein. The initial translation product is a large precursor which can be divided into four contiguous regions (pro, α, β and γ). The pro region encodes a polypeptide of 25,600 Da. The propeptide is absent from the final secreted protein. Activation of the precursor is achieved by autolysis or through the action of a second enzyme as part of the secretion process.

PrpRI is readily released from the surface of all *P. gingivalis* strains examined to date but is absent from other related oral and intestinal anaerobic bacteria [9]. High concentrations of PrpRI are found associated with the outer membrane of *P. gingivalis* and are therefore thought to be responsible for the hemagglutinating activity of the bacterial vesicles [10]. The β component of the ArgI dimer contains a number of features typical of adhesins and binding proteins. For example there are regions on the PrpRI which have similarities to the circumsporozoite protein of *Plasmodium falciparum* which is involved in attachment of the parasite to hepatocytes [6].

Thus PrpRI consists of two domains, an N-terminal proteolytic domain and a C-terminal hemagglutinin domain. Work carried out by Aduse-Opoku *et al.* [6] on characterisation of the PrpRI protease, showed that the arginine-specific protease migrated as a 50 kDa band on SDS-PAGE gels. The hemagglutinin domain has also been shown to migrate as a 50kDa band on SDS-PAGE and it is to this region that the 50kDa photo-labile protein (A) corresponds.

The third photo-labile protein (protein C) that we identified was the lysine-specific cysteine proteinase, KGP, of *P. gingivalis*. Like PrpRI, KGP not only possesses proteolytic activity but also has adhesive domains [7].

The second photo-labile protein (protein B) that we detected could not be identified unequivocally as it was identical to regions in PrpRI and KGP. In both cases these regions corresponded to the hemagglutinin domain.

The finding that photosensitization destroys the proteolytic domain of at least one of major proteases pro-

duced by *P. gingivalis*, KGP, explains our previous observations that treatment of this organism with a photosensitizer and laser light resulted in a reduction of the proteolytic activity associated with this bacterium [8]. In addition to this, photosensitization also destroys the hemagglutinin domain of at least one of the major proteases, PrpRI, and may also attack that of KGP. The fact that lethal photosensitization adversely affects enzymes which are involved in the destruction of host connective tissue and disruption of normal host defense mechanisms leads to the inference that lethal photosensitization (or photodynamic therapy [PDT] when applied clinically) can be used to hinder colonization of bacteria within the periodontal pocket and, more importantly, to reduce damage to the host. Conventional therapies for periodontitis, such as antibiotic therapy and mechanical removal of the dental plaque, rely on eliminating or removing the causative pathogen from the periodontal pocket in order to eradicate the disease. As mentioned above, work has shown that the enzymes required for colonization and host tissue destruction are present on the surface of *P. gingivalis* and in the surrounding tissue. Therefore, unlike conventional treatments, PDT is capable of not only causing reductions in the bacterial load, but also neutralizing key virulence factors present after the destruction of the bacteria. In conclusion, the results of this study have identified proteases of *P. gingivalis* as major targets for the cytotoxic species generated during lethal photosensitization of the organism using TBO and red light.

ACKNOWLEDGMENTS

The authors wish to acknowledge the Charles Wolfson Charitable Trust for providing the funding for this work.

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