Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 673–681

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01389.x

Characterization of human-type monoclonal antibodies against reduced form of hemin binding protein 35 from *Porphyromonas gingivalis*

Y. Shibata[1,2], S. Okano[1,2], T. Shiroza[2,3], T. Tahara[4], K. Nakazawa[4], S. Kataoka[4], I. Ishida[4], T. Kobayashi[5], H. Yoshie[5], Y. Abiko[1,2]

[1]Department of Biochemistry and Molecular Biology, [2]Research Institute of Oral Science and [3]Department of Chemistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan, [4]Kyowa Hakko Kirin Co. Ltd, Tokyo, Japan and [5]Division of Periodontology, Department of Oral Biological Science, Niigata University, Graduate School of Medical and Dental Sciences, Niigata, Japan

Shibata Y, Okano S, Shiroza T, Tahara T, Nakazawa K, Kataoka S, Ishida I, Kobayashi T, Yoshie H, Abiko Y. Characterization of human-type monoclonal antibodies against reduced form of hemin binding protein 35 from Porphyromonas gingivalis. *J Periodont Res 2011; 46: 673–681. © 2011 John Wiley & Sons A/S*

Background and Objective: The gram-negative anaerobe *Porphyromonas gingivalis* has been implicated as an important pathogen in the development of adult periodontitis, and its colonization of subgingival sites is critical in the pathogenic process. We previously identified a 35 kDa surface protein (hemin binding protein 35; HBP35) from *P. gingivalis* that exhibited coaggregation activity, while additional analysis suggested that this protein possessed an ability to bind heme molecules. For development of passive immunotherapy for periodontal diseases, human-type monoclonal antibodies have been prepared using HBP35 as an antigen in TransChromo mice. In the present study, we focused on a single antibody, TCmAb-h13, which is known to inhibit heme binding to recombinant HBP35. The aim of our investigation was to clarify the redox-related function of HBP35 and consider the benefits of human-type monoclonal antibodies.

Material and Methods: To examine the antigen recognition capability of TCmAbs with immunoblotting and Biacore techniques, we used the native form as well as several Cys-to-Ser variants of recombinant HBP35.

Results: We found that the redox state of recombinant HBP35 was dependent on two Cys residues, ^{48}C and ^{51}C, in the thioredoxin active center (WCGxCx). Furthermore, TCmAb-h13 recognized the reduced forms of recombinant HBP35, indicating its inhibitory effect on *P. gingivalis* growth.

Conclusion: Hemin binding protein 35 appears to be an important molecule involved in recognition of the redox state of environmental conditions. In addition, TCmAb-h13 had an inhibitory effect on heme binding to recombinant HBP35, thereby interfering with *P. gingivalis* growth.

Dr Yoshimitsu Abiko, DDS, PhD and Yasuko Shibata, PhD, Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, 2-870-1, Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan
Tel: +81 47 360 9332
Fax: +81 47 360 9329
e-mail: abiko.yoshimitsu@nihon-u.ac.jp; shibata.yasuko@nihon-u.ac.jp
Present address: Isao Ishida, Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Chiba, Japan.

Key words: hemin binding protein; *Porphyromonas gingivalis*; redox; thioredoxin domain

Accepted for publication May 14, 2011

Periodontitis, one of the most common oral diseases in humans, is induced by infection with the anaerobic gram-negative bacterium *Porphyromonas gingivalis*. Various types of organisms are commonly isolated from subgingival microflora (1), and *P. gingivalis* has been recognized as a major pathogen of adult periodontitis (1,2). Possible invasive routes into host tissues include coaggregation with oral microflora, such as *Streptococcus gordonii*, hemagglutination with erythrocytes followed by uptake of iron atoms, and attachment to host epithelial cells. Hence, the proteins involved in each step are recognized to be virulence factors originating from the bacterium. Our approach for disease prevention is to establish a passive immunization system by which endogenous antibody molecules can be directly applied to infected tissue (3). For this goal, several of the virulence factors mentioned above were examined (4,5). Among those, we previously focused our attention on a 35 kDa surface protein expressed by *P. gingivalis* that is involved not only in coaggregation with oral microflora (6–8) and hemagglutination of erythrocytes, but also in binding with heme molecules (9). For this reason, this protein is now referred to as hemin binding protein 35, or HBP35. In addition, nucleotide sequencing of the gene coding for this protein (*hbp35*) revealed the presence of a catalytic center for thioredoxin. The amino-acid stretch Cys-x-x-Cys is essential for enzymes possessing thioredoxin activity (10–12), because disulfide bond formation by nonenzymatic air oxidation is relatively inefficient, and organisms ranging from bacteria to humans are equipped with enzyme systems to catalyse this process. The reversible redox reaction occurring at the Cys residues provides chemical potential for a number of biological reactions. We further characterized HBP35 by amino-acid substitution, and our findings clearly suggested that it is involved in disulfide bond formation in *P. gingivalis* (13).

Tomizuka *et al.* (14) developed TransChromo (TC) technology, which enables introduction of megabase-sized segments of DNA into cells. They have used this approach to develop mice that carry megabases of human DNA by utilization of a human chromosome fragment as a vector. TransChromo technology has been applied to development of the TC Mouse™, which incorporates entire human immunoglobulin (hIg) loci. The TC Mouse™ expresses a fully diverse repertoire of human immunoglobulins, including all subclasses of IgGs (IgG1–IgG4). Furthermore, immunization of the TC Mouse™ with various human antigens has produced antibody responses comprised of human antibodies. Using such mice, we have obtained several TC Mouse™-derived monoclonal antibodies (TCmAbs), which have shown different characteristics in terms of binding to recombinant HBP35 (rHBP35) proteins. For example, TCmAbs promoted the phagocytosis of *P. gingivalis* by neutrophils (15), further indicating their protective effect against alveolar bone loss due to oral infection with *P. gingivalis* (16). Together, these results suggest that TCmAbs against the surface membrane protein of *P. gingivalis* HBP35 can be used to protect against periodontal diseases caused by *P. gingivalis* infection. In our analysis of these TCmAbs with regard to their antigen-binding abilities, we found different types of antibodies in the TCmAbs library, which were designated as TCmAb-a44, TCmAb-h13, TCmAb-1-85 and TCmAb-5-89. In the present study, we focused on a single TCmAb, TCmAb-h13.

Material and methods

Purification of rHBP35 and Cys-modified rHBP35 proteins

Recombinant HBP35 from *P. gingivalis* 381 was purified from cell sonicates of an *Escherichia coli* K-12 strain harboring the recombinant plasmid pMD125, as previously described (8). *E. coli* clone cells were cultured using a method previously reported (7). The *E. coli* supernatant was dialysed with distilled water overnight, then purified using a Source 15Q column (ÄKTA System, GE Healthcare, Little chalfont, UK). The recombinant protein fraction was then further purified by electro-osmotic preparative disc sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 17). To detect this molecule, anti-HBP35 rabbit serum was used. Eluted fractions were then dialysed with distilled water overnight.

Hemin binding protein 35 contains a total of four Cys residues at amino-acid positions 48, 51, 113 and 161, and we attempted to substitute these residues with Ser residues. Six kinds of Cys residue-mutated rHBP35 proteins (^{48}Cys to Ser, ^{51}Cys to Ser, ^{113}Cys to Ser, ^{161}Cys to Ser, both ^{48}Cys and ^{51}Cys to Ser, and both ^{113}Cys and ^{161}Cys to Ser) were constructed using the method of Shiroza *et al.* (13). The Cys-modified rHBP35 proteins were purified to near homogeneity from sonicates of *E. coli* transformants harboring the respective plasmids, as previously reported (8).

Replacement of Cys with Ser in purified rHBP35 and the six varieties of Cys-mutated rHBP35s was then confirmed using a MALDI TOF-MS system (Shimadzu Biotech, Kyoto, Japan). Recombinant HBP35 and its variant proteins were semi-automatically excised and treated with trypsin using Xcise, a high-throughput gel-excise processor (Shimadzu Biotech; 18). Peptide mass analysis was carried out with an AXIMA CFR MALDI-TOF mass spectrometer (Shimadzu Biotech). Proteins were identified from peptide mass data using the database search engine MASCOT (Matrix Science, Boston, MA, USA), with a database for HBP35 that included mutagenic protein sequences.

Screening of antibodies

Mice from the TC Mouse™ line (14) were immunized with rHBP35 as an antigen, and several human TC antibodies (TCmAbs) against rHBP35 were prepared (15). First, hybridoma clone selection was confirmed by ELISA. After coating 96-well plates with the antigen (purified rHBP35, 50 ng per well; *P. gingivalis* cell suspensions, 2.5 μg per well), culture media of hybridoma cells containing TC antibodies were incubated with 5% milk, then treated with secondary

anti-human antibodies. Coaggregation was examined by the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus* whole cells using the method of Hiratsuka *et al.* (7). Phagocytosis was determined based on neutrophils (%) induced by *P. gingivalis* phagocytosis (19). Based on the anti-coaggregation and neutrophil phagocytotic effects against *P. gingivalis*, human monoclonal antibodies were selected and purified using a Protein A column. A TCmAb generated from a TC Mouse™ immunized with 2,4-dinitrophenol conjugated to keyhole limpet hemocyanin was used as a control (TCmAb-cIgG).

Preparation of anti-rHBP35 polyclonal antibody

Anti-rHBP35 rabbit antiserum (PAb-rHBP35) was prepared by immunizing New Zealand White rabbits with purified rHBP35, after which the purified IgG fragment was isolated on a Protein G column, followed by further purification using rHBP35 affinity column chromatography.

Immunological analysis

Native rHBP35 and Cys-mutated variants were subjected to SDS–PAGE, then transferred to a nitrocellulose membrane in transfer buffer (20% methanol and 50 mM Tris–glycine; 1 h at 6 V). The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline (10 mM Tris–HCl buffer with 0.9% NaCl, pH 7.4) for 30 min at room temperature, after which it was incubated first with one of the TCmAbs for 16 h, then with peroxidase-conjugated goat anti-human IgG (1:1000 dilution) in blocking buffer for 1 h at room temperature. The TCmAb-positive protein bands on the nitrocellulose membranes were detected by incubation with 0.1 M Tris–HCl (pH 7.4) containing 0.08% 3,3′-diaminobenzidine tetrahydrochloride and 0.05% H_2O_2.

For analysis of the isoelectric point of HBP35, a Mini EF Cell system (model 111, Bio-Rad Laboratories Inc., Hercules, CA, USA) was used. Polyacrylamide gels including 40% ampholine solution (pH 3.5–10.0) were prepared and supported by GelBond PAG (FMC BioProducts, Rockland, ME, USA). After electrophoresis, the gels were carefully removed from the GelBond and stained with Coomassie brilliant blue (CBB).

Binding assay using Biacore system

Binding of TCmAbs to rHBP35 was analysed using a model 2000 system from Biacore AB (Biacore Co., Uppsala, Sweden). Recombinant HBP35 and Cys-mutated rHBP35 were immobilized on a CM5 sensor chip, using an amine coupling kit containing *N*-hydroxysuccinimide, *N*-ethyl-*N*′-[(3-dimethylamino)-propyl]-carbodiimide hydrochloride and ethanolamine–HCl. The TCmAbs were dissolved in HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% surfactant P20) and injected at a flow rate of 10 μL/min. Regeneration was performed using a 10 mM glycine–HCl buffer (pH 1.5). As a control antibody, TCmAb-cIgG was used.

P. gingivalis culture and growth-inhibitory effects of TCmAbs

P. gingivalis 381 was purchased from the Japan Collection of Microorganisms, RIKEN (Institute of Physical and Chemical Research, Saitama, Japan). The organisms were grown in Todd–Hewitt broth, supplemented with hemin (5 μg/mL) and vitamin K1 (0.01%), in an anaerobic atmosphere (80% N_2, 10% H_2 and 10% CO_2). To check the inhibitory effects of *P. gingivalis* growth by TCmAbs, bacterial cells were subcultured in hemin-limited medium (0.01 μg/mL). After an

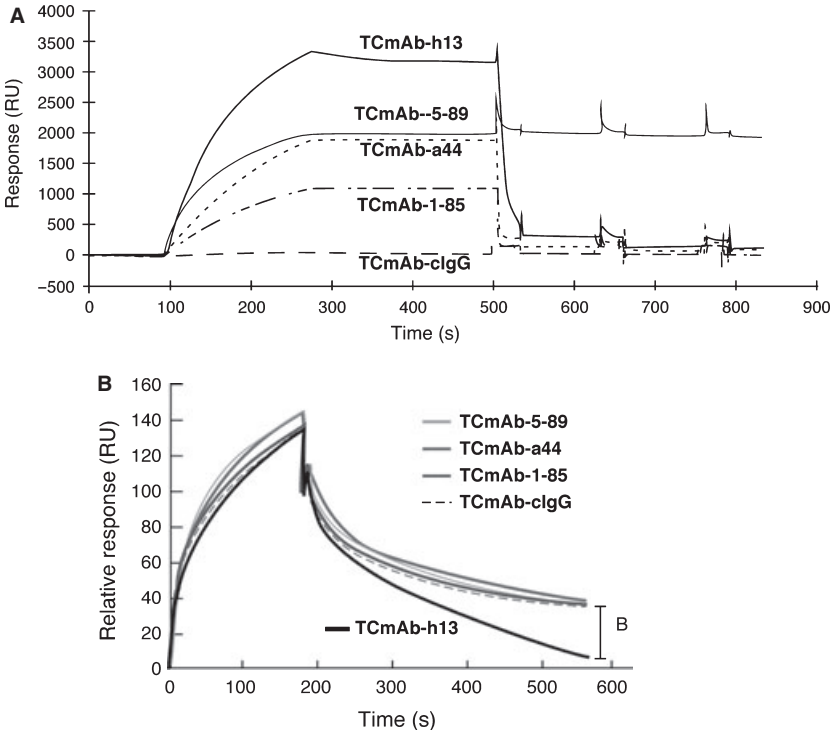

Fig. 1. Binding ability of TCmAbs to rHBP35 protein. (A) The binding of TCmAbs was investigated using a Biacore 2000 system. TCmAb-h13, TCmAb-a44, TCmAb-5-89 and TCmAb-1-85 were applied to a Biacore CM5 chip, which immobilized rHBP35. The TCmAbs used as analytes for binding to the antigen were purified using a protein G column and resolved in HBS-EP buffer to 200 nM. Eighty milliliters of each TCmAb solution was associated with the immobilized rHBP35 protein as a ligand on the CM5 chip for 3 min (10 μL/min), then dissociated using HBS-EP buffer without antibodies for 2 min (10 μL/min). Regeneration was performed three times using a 10 mM glycine–HCl buffer (pH 1.5). (B) The anti hemin binding of TCmAbs was determined. After applying each TCmAb for 2 min, hemin (5 μg/mL) was added. RU, resonance unit.

additional subculture in hemin-limited medium for 24 h, *P. gingivalis* cells (50 μL) were treated with 100 μL of the TCmAbs (1.5 mg/mL) for 30 min. Next, hemin-supplemented medium (5 mL) was added and the cells were cultured in an anaerobic atmosphere. The growth level of *P. gingivalis* cells was determined by turbidity at 550 nm.

Statistical analysis

For growth inhibition, data are presented as means ± SD of triplicate assays. The difference between the means of two independent samples in the experiment was determined using the Student's unpaired *t*-test. A *p*-value of ≤ 0.05 was considered significant.

Results

Selection of hybridoma clone for anti-hemin binding

After screening for the ability of antigen recognition using rHBP35-coated 96-well plates, several hybridoma clones were cultured, and TCmAbs produced in the cultured media were purified. Based on their binding abilities to rHBP35, as shown by ELISA, the anti-coaggregation effects and neutrophil phagocytotic effects against *P. gingivalis* of four human monoclonal antibodies, designated as TCmAb-a44, TCmAb-h13, TCmAb-5-89 and TCmAb-1-85, were analysed (15). To confirm their binding abilities to the rHBP35 protein, a Biacore 2000 system utilizing a CM5 sensor chip immobilized with 3161 resonance unit (RU) of rHBP35 was employed. After association with the antibodies for 2 min, HBS-EP buffer without TCmAbs was applied to the sensor chip to dissociate the bound molecules. As shown in Fig. 1A, TCmAb-h13 showed the highest level of binding against rHBP35, while the dissociation levels of all the TCmAbs appeared to be high. Both TCmAb-a44 and TCmAb-5-89 also showed high levels of association against HBP35 molecules (TCmAb-h13 > TCmAb-a44 ≥ TCmAb-5-89 > TCmAb-1-85), while binding to rHBP35 was not shown by the control TCmAb-cIgG (15).

Using the same CM5 sensor chip immobilized with rHBP35, the inhibitory effects of TCmAbs for hemin binding were examined. After applying each antibody (20 μg/mL, 20 μl/min), hemin solution (5 μg/mL, 20 μL/min) was applied to the chip. Interestingly, TCmAb-h13 had greater anti-hemin-binding effects (ΔB) compared with TCmAb-cIgG, while the other TCmAbs had no effects (Fig. 1B).

Molecular characterization of rHBP35

A computer-aided motif search indicated that HBP35 was composed of several functional domains, including the most prominent amino-acid stretch, WCGYCP, which was identified near the N-terminal end, whereas the conserved motif of the active center, WCGxCx, has been found in many thioredoxins (13; Fig. 2A).

As shown in Fig. 2B, a few protein bands were recognized by immunoblotting in a pI range of 6.5–7.5 when purified rHBP35 was subjected to isoelectric focusing. Net charges were calculated from the contents of dibasic and dicarboxylic amino acids, which were expected to have pI values of about 6.64 (Genetyx-Mac, Co., Osaka Japan). Indeed, rHBP35 was focused near the pI point on the gel near as a few bands recognized by polyclonal antibody (PAb-rHBP35). Furthermore, purified rHBP35 was incubated in the presence (+) or absence (−) of β-mercaptoethanol (MeSH) for 1 min at 100°C and subjected to SDS–PAGE (Fig. 2C).

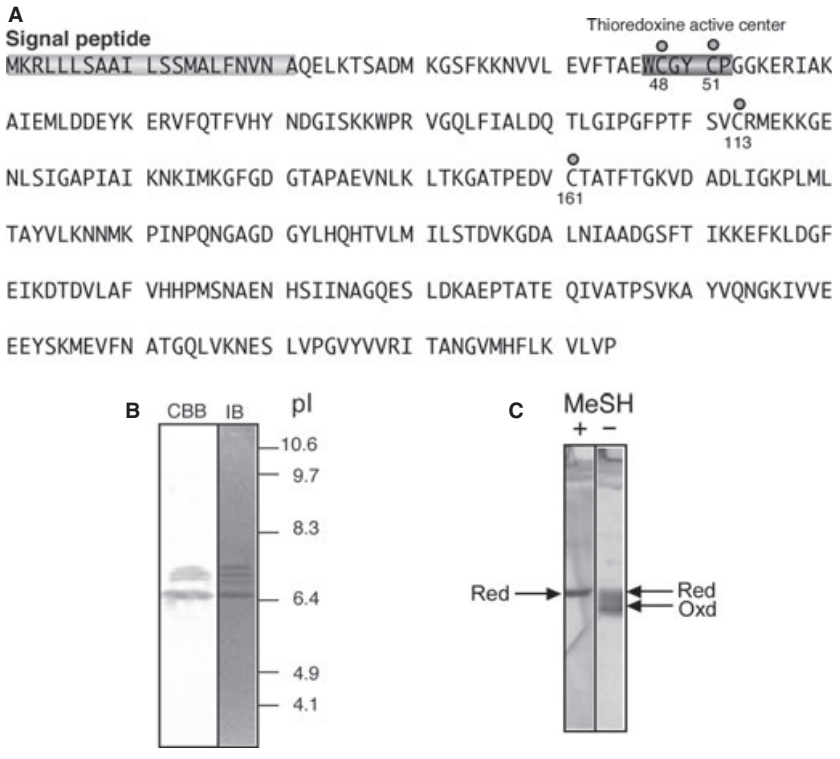

Fig. 2. Cys position in HBP35 and redox-related character of native rHBP35. (A) Amino acid sequence of HBP35. Four Cys residues in the HBP35 protein are marked by circles with an amino-acid co-ordinate number. The conserved amino-acid stretch, WCGYCP, a typical active center found in thioredoxin, is indicated in the amino-acid sequence of HBP35. (B) Immunoblot analysis of isoelectric electrophoresis of purified rHBP35. The gel was stained with Coomassie brilliant blue (CBB). rHBP35 on the gel was transferred onto a nitrocellulose membrane and incubated with pAb-rHBP35. IB, immunoblotting with affinity column-purified anti-rHBP35 polyclonal antibody (pAb-rHBP35). (C) Effect of β-mercaptoethanol (MeSH) on native rHBP35. Purified rHBP35 was incubated in the presence (+) or absence (−) of MeSH for 1 min at 100°C and subjected to SDS–PAGE. After electrophoresis, proteins were stained with CBB.

After electrophoresis, proteins were stained with CBB. When the disulfide bridges of the recombinant protein were reduced by MeSH, only a single band corresponding to the reduced form of rHBP35 was observed in the former conditions. In the latter environment, two rather obscure bands were detected, of which the smaller band seemed to be the oxidized from.

Redox state of rHBP35 and its recognition by TCmAbs

As the molecular characteristics of rHBP35 appeared to be influenced by the redox state of the protein, we next focused on the redox-related amino-acid residues within rHBP35, in particular Cys. The HBP35 protein has four Cys residues, ^{48}Cys, ^{51}Cys, ^{113}Cys and ^{161}Cys, and the active center of thioredoxin is formed by ^{48}Cys and ^{51}Cys, as indicated in Fig. 2A. We investigated the binding abilities of TCmAb-h13 and TCmAb-1-85 against Cys-mutated rHBP35 proteins using immunoblot analysis. We additionally prepared Cys-mutated rHBP35, ^{48,51}Ser-rHBP35 and ^{113,116}Ser-rHBP35. Even on the SDS–PAGE without MeSH, ^{48}Ser-rHBP35, ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35 appeared in a reduced form, while rHBP35, ^{113}Ser-rHBP35, ^{161}Ser-rHBP35 and ^{113,161}Ser-rHBP35 were observed in both oxidized and reduced states on the CBB-stained gel (Fig. 3A, top). In the same conditions, no binding abilities for TCmAb-1-85 to ^{48}Ser-rHBP35 (lane 1), ^{51}Ser-rHBP35 (lane 2) or ^{48,51}Ser-rHBP35 (lane 5) were found [Fig. 3A, MeSH(−)]. As the Cys residues that form the thioredoxin active center in HBP35 might be important for maintaining the oxidized form of this molecule in aerobic conditions and two of the Cys residues from mutated rHBP35 were in the thioredoxin active centre, ^{48}Ser-rHBP35, ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35 were found only in a reduced form in the SDS–PAGE gels, even without MeSH, as shown in Fig. 3A. The TCmAb-1-85 should have recognized the oxidized form of rHBP35, but not the reduced form of the thioredoxin domain, while TCmAb-h13 showed contrasting

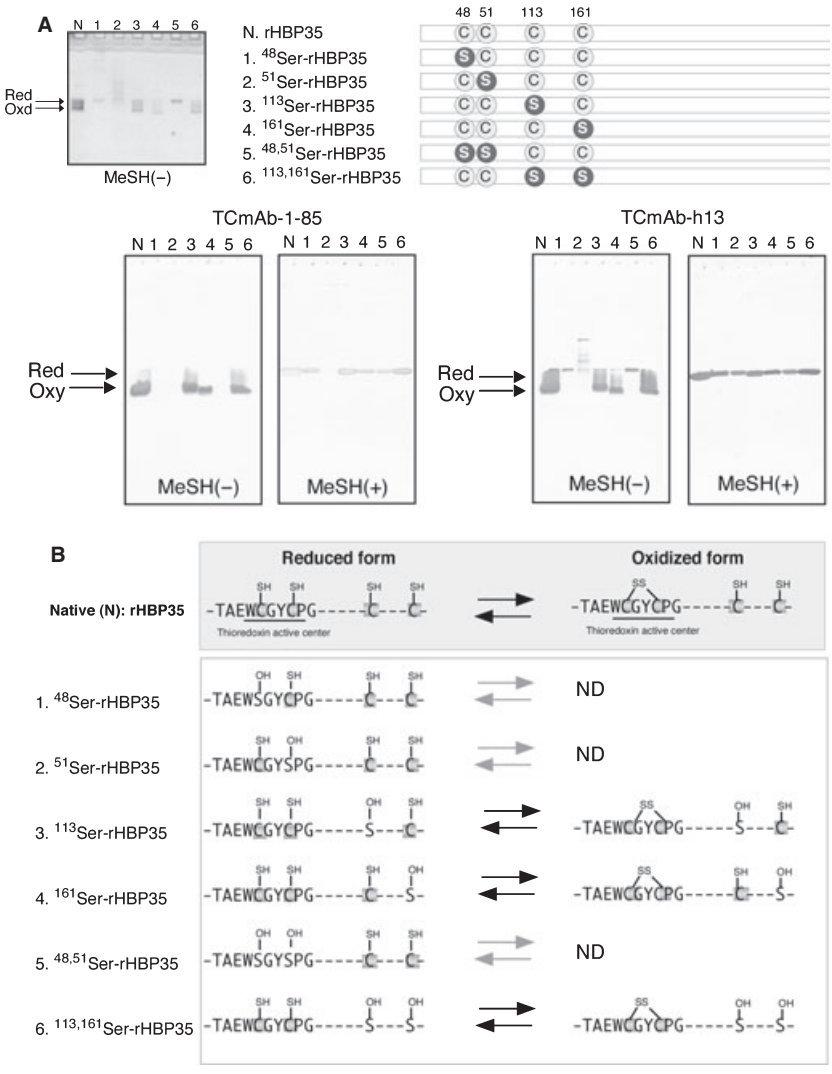

Fig. 3. Redox state of native and variant rHBP35 proteins. (A) SDS–PAGE and immunoblot analyses of native and variant rHBP35 proteins. Native (lane N) and variant rHBP35 proteins (lanes 1–6) were subjected to SDS–PAGE without MeSH incubation, and stained with CBB (upper gel). Native and variant rHBP35 proteins were incubated with or without MeSH, resolved on SDS–PAGE, and analysed with TCmAb-1-85 and TCmAb-h13 following transfer onto a nitrocellulose membrane (lower panels). (B) Possible HBP35 protein structure around the Cys residues depending on redox state.

results. For further elucidation, the redox-related properties of native rHBP35 and the Cys-mutated variants are summarized in Fig. 3B. It seemed that both ^{48}Cys and ^{51}Cys were important for maintaining rHBP35 in an oxidized form in aerobic conditions. Furthermore, we succeeded in constructing TCmAbs that specifically recognized the oxidized form of rHBP35. Moreover, it is interesting that the anti-hemin-binding TC antibody, TCmAb-h13, recognized the reduced form of rHBP35.

In order to gain a better understanding of the binding of antibodies to HBP35, we analysed the binding variation of Cys-mutated rHBP35 using a Biacore system. A Biacore system utilizing a CM5 sensor chip immobilized with approximately 3000 RU each of ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35 was employed. We found binding activities of TCmAb-h13 and TCmAb-a44 against Cys-mutated rHBP35, ^{48}Ser-rHBP35 and ^{48,51}Ser-rHBP35, while TCmAb-1-85 was found to have no such binding ability (Fig. 4).

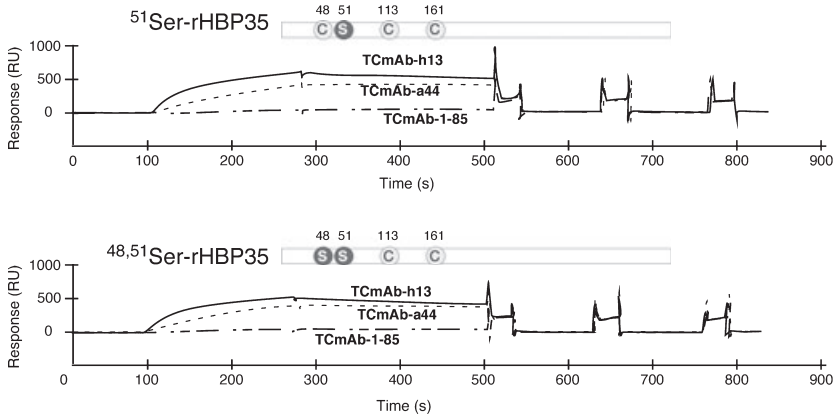

Fig. 4. TCmAb binding to thioredoxin domain of variant rHBP35 protein. ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35 were immobilized on a sensor chip CM5 using an amine coupling kit. TCmAb-h13, TCmAb-a44 and TCmAb-1-85 were dissolved in HBS-EP and injected at a flow rate of 20 μL/mL. Regeneration was performed using a 10 mM glycine–HCl buffer (pH 1.5).

Inhibitory effects of TCmAbs on *P. gingivalis* cell growth

To determine cell growth in anaerobic conditions, *P. gingivalis* cells were cultured in hemin-limited medium, then further treated with each antibody for 30 min and transferred to hemin-supplemented medium. Growth inhibition was preliminarily observed in the cultivation period from 22 to 26 h (data not shown). As shown in Fig. 5, TCmAb-h13 significantly exhibited inhibitory activity after 22 h of incubation.

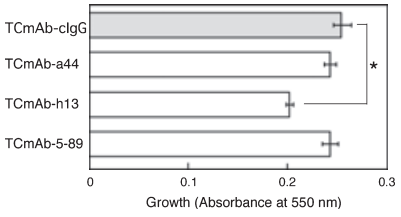

Fig. 5. Inhibitory effect of TCmAbs on *Porphyromonas gingivalis* growth. The growth of *P. gingivalis* cells with or without TCmAbs was assessed. After subculturing twice in hemin-limited medium, *P. gingivalis* cells were incubated with or without TCmAb-a44, TCmAb-h13 or TCmAb-5-89 for 30 min. Fresh medium supplemented with hemin was added to the culture, and incubation was continued for 22 h in an anaerobic atmosphere. To quantify the inhibitory effects of TCmAbs on *P. gingivalis* growth, cell density at 22 h was determined and compared. $*P < 0.05$, $n = 3$.

Inhibitory effect of TCmAbs on hemin binding to rHBP35

Finally, we examined the effects of anti-rHBP35 mAbs on hemin–rHBP35 binding using a Biacore system. In this experiment, we analysed the interactions between hemin and rHBP35, as well as between hemin and TCmAb and between TCmAb and rHBP35. We first prepared TCmAbs immobilized on CM5 chips to assess their inhibitory effects on hemin–rHBP35 binding. After immobilization of the Abs, 80 μL of 5 μM rHBP35 protein solution was infused for 2 min at 10 μL/min, after which 12.5 μM of hemin solution was infused for 2 min. As shown in Fig. 6, rHBP35 bound to TCmAb-1-85 on the CM5 chip with slow association–dissociation kinetics (thick dashed line), thus the RU values attributable to this interaction remained constant throughout the observation period. Infusion of 12.5 μM of hemin solution elicited a further increase in the RU values (thick continuous line), indicating that TCmAb-1-85, which binds to the oxidized form of the protein, does not interfere with hemin–rHBP35 binding. In contrast, the binding of rHBP35 to TCmAb-h13 resulted in faster association–dissociation kinetics, and bound rHBP35 was quickly released when hemin was supplied, suggesting a competitive interaction between rHBP35 and hemin for binding to TCmAb-h13 (Fig. 7). This competitive interaction was confirmed when we examined the effects of TCmAb-h13 on hemin–rHBP35 binding using a CM5 chip upon which rHBP35 was immobilized.

As shown in Fig. 7, inhibition of the interaction between hemin and rHBP35 by TCmAb-h13 was determined by the signal produced by TCmAb-h13 + hemin (ΔA), which was substantially smaller, indicating that TCmAb-cIgG + hemin binding (ΔB) to rHBP35 was comparatively weak. In addition, the binding signal obtained at 380 s, when TCmAb-h13 and hemin were applied simultaneously, should have been smaller than the sum of the signals obtained when a control antibody (TCmAb-cIgG) + hemin were separately applied. The TCmAb- h13 + hemin binding signal was approximately the same as the TCmAb-h13 signal after diffusion of the analytes, while the TCmAb-cIgG + hemin binding signal was similar to those seen with hemin binding (ΔC).

Discussion

We have constructed several TCmAbs utilizing the TC Mouse™ with rHBP35 as an antigen. In this study, we selected four representative TCmAbs, namely TCmAb-a44, TCmAb-h13, TCmAb-1-85 and TCmAb-5-89, and focused on the antigen-binding abilities/characteristics of these antibodies to the rHBP35 protein. The present data clearly indicate that TCmAb-h13 recognized the reduced form of rHBP35.

Using immunoblot analysis with TCmAbs, we initially investigated the capacity of the rHBP35 variants to produce a redox state. All proteins were prepared in the absence of MeSH and then subjected to SDS–PAGE. The Cys residues forming the thioredoxin active center in HBP35 appeared to be critical for maintaining the molecule in an oxidized form, thus ^{48}Ser-rHBP35, ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35 were found only in a reduced form, even without MeSH (Fig. 3A, top). Also,

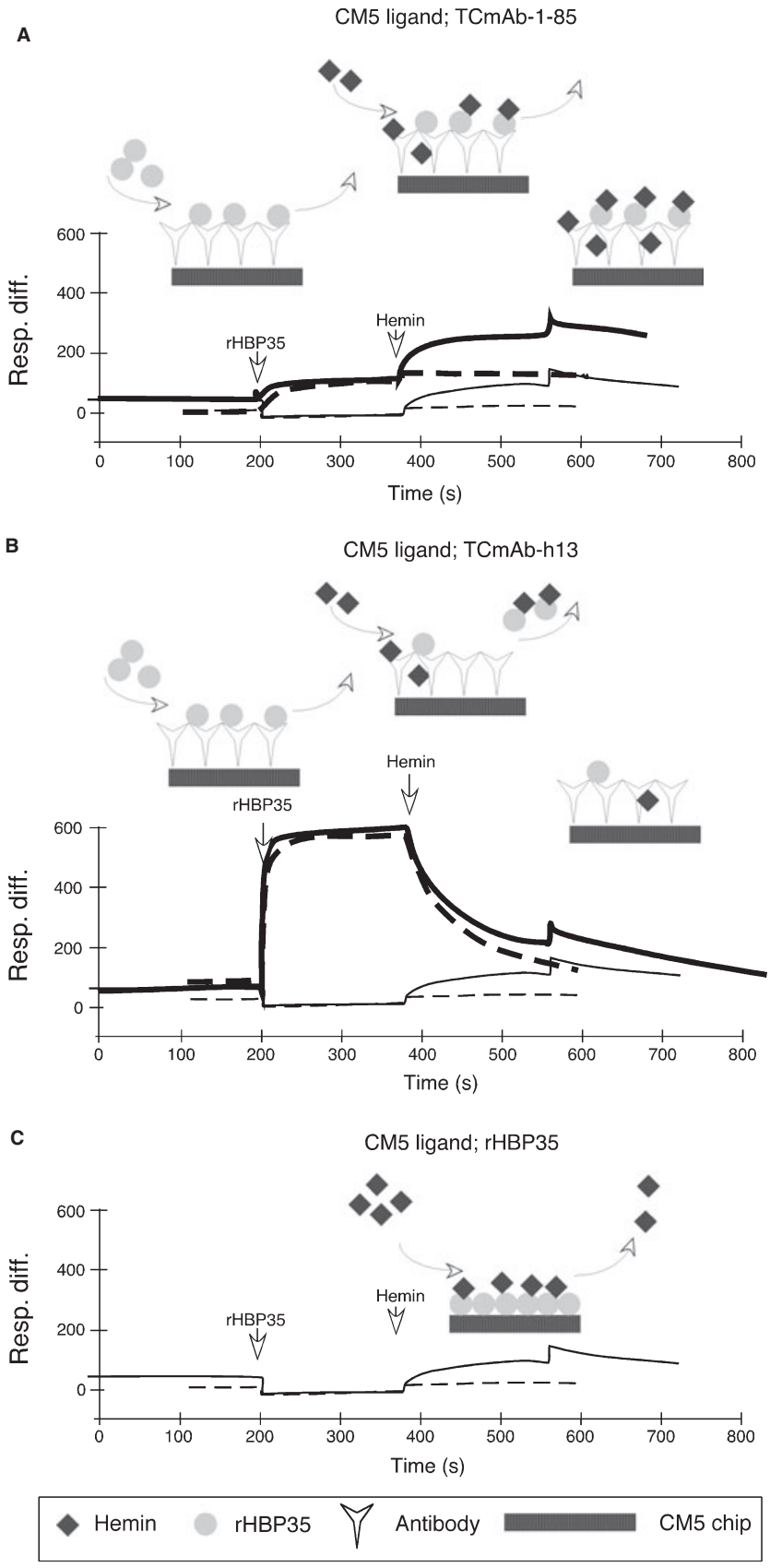

Fig. 6. Interactions among rHBP35, TCmAbs and hemin. The binding of TCmAb-1-85 (A) or TCmAb-h13 (B) to rHBP35 and the inhibitory effects on hemin binding to rHBP35 were analysed using a Biacore 2000. rHBP35 (5 μM, 10 μL/min) was applied to the Biacore chip for 3 min, after which a hemin solution (12.5 μM, 10 μL/min) was applied for an additional 3 min. Thin lines in (A) and (B) show the results of rHBP35 (C), while thick lines show rHBP35 binding to TCmAbs on the CM5 chip. The dashed lines show binding of rHBP35 protein without additional binding by hemin to the TC antibodies (thick dashed line) and rHBP35 (thin dashed line). rHBP35, TCmAb-h13 and TCmAb-1-85 were immobilized on the CM5 chip at 3075, 2328 and 2064 RU, respectively.

TCmAb-1-85 did not bind to the Cys-mutated rHBP35s, ^{48}Ser- rHBP35, ^{51}Ser-rHBP35 and ^{48,51}Ser-rHBP35, which implies that TCmAb-1-85 recognizes a disulfide bond between the two Cys residues in the thioredoxin active center of rHBP35. In contrast, TCmAb-h13 recognized the reduced form of rHBP35. Taken together, these results suggest that the redox state of rHBP35 is dependent on the two Cys residues, ^{48}Cys and ^{51}Cys, in the thioredoxin active center (WCGxCx) of this molecule, and that we succeeded in constructing two TC antibodies, TCmAb-1-85 and TCmAb-h13, which recognized the oxidized and reduced forms, respectively, of rHBP35. However, this conclusion raises questions regarding the SDS–PAGE profile shown in Fig. 3, because the reduced form of rHBP35 was recognized by TCmAb-1-85. It is likely that during manipulation of the acrylamide gel, air oxidation occurred, which resulted in conversion of the reduced protein into an oxidized form. In contrast, TCmAb-h13 demonstrated an ability to bind to both reduced and oxidized proteins (Fig. 3A). The present data clearly indicate that TCmAb-1-85 and TCmAb-h13 recognized the oxidized and reduced forms, respectively, of rHBP35.

In contrast, TCmAbs-h13 was selected for its hemin-binding ability during the screening process for hybridomas (Fig. 1B). Specifically, TCmAb-

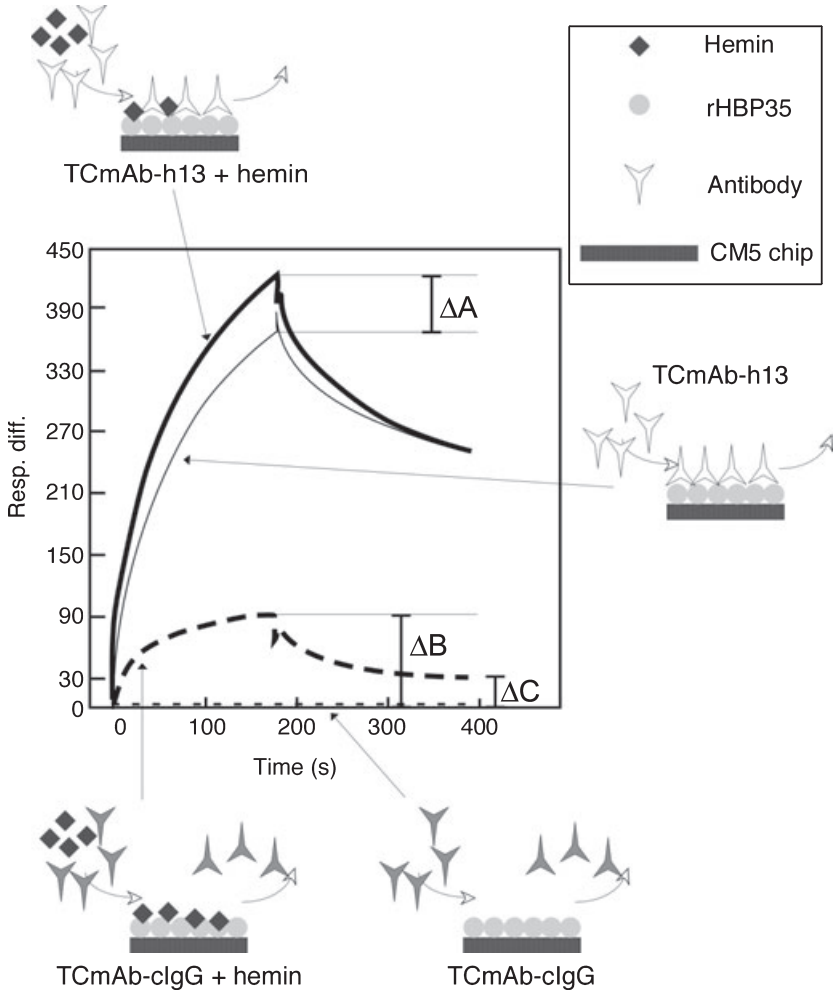

Fig. 7. Inhibitory effect of TCmAb-h13 on hemin binding to rHBP35. The inhibitory effect of TCmAb-13 on hemin binding to rHBP35 was analysed using a CM5 chip, upon which rHBP35 was immobilized. TCmAb-13 alone (thin continuous line), TCmAb-h13 + hemin (thick continuous line), TCmIgG alone (thin dashed line) and control IgG + hemin (thick dashed line) were applied to an rHBP35-immobilized chip for 3 min (5 μM, 20 μL/min). rHBP35 was immobilized on the CM5 chip at 3500 RU.

h13 showed inhibitory effects on hemin binding to rHBP35, while no effect was seen with TCmAb-cIgG (Fig. 6).

It is known that *P. gingivalis* has an obligate requirement for hemin or selected heme- or iron- containing compounds for its growth (20–22). In addition, several oral bacteria can generate products that make the microfloral environment more oxidative or reductive, which could potentially influence the ecology of subgingival sites (23,24). Therefore, we focused our attention on *P. gingivalis* HBP35, since this protein is involved not only in co-aggregation with other oral microflora, but is also important for heme binding (6–9). In addition, we further characterized the functions of the Cys residues in this protein (9,13). Although the heme-binding mechanisms and roles of the gram-negative anaerobe *P. gingivalis* have not yet been clarified, heme binding and uptake are considered to be fundamental factors for both its growth and virulence. Recently, Shoji *et al.* (25) reported that HBP35 plays a role in hemin utilization with sufficient hemin concentration by assessing the growth of an *hbp35*-deleted mutant of *P. gingivalis*. Therefore, we determined the inhibitory effects of TCmAbs on the growth of *P. gingivalis* cells by incubating those cells with each of the individual TCmAbs. As shown in Fig. 5, TCmAb-h13 significantly exhibited inhibitory activity after 22 h of incubation, hemin binding to the recombinant antigen protein on the Biacore chip. Although this confirmed that TCmAb-h13 recognized the reduced state of rHBP35, thereby interfering with hemin binding, it remains unclear whether this TCmAb directly recognizes the rHBP35 Cys residues.

Oxidative stress has been defined as a disturbance in the pro-oxidant–antioxidant balance in favor of pro-oxidants and has been reviewed, along with oxygen metabolism, in regard to its relationship with dental plaque biofilm formation (26). Heme binding and uptake are thought to be fundamental factors for both the growth and the virulence of *P. gingivalis*. Interestingly, it has been reported that when grown in the presence of a high concentration of hemin, *P. gingivalis* can tolerate even 10% oxygen because of the protective effect provided by hemin (27). Thus, HBP35 is likely to be a multifunctional molecule, mediating heme uptake as well as serving as a hemoprotein that changes its structure with the reduction/oxidation of iron/hemin. We speculated that HBP35 serves as an oxygen sensor in *P. gingivalis* and that heme serves as an important cofactor, acting as a redox-controlled molecular switch. In addition, it is suggested that this enables HBP35 to serve as an oxygen sensor in *P. gingivalis* that recognizes the environmental redox state on the bacterial surface via the two thiol groups in its thioredoxin active center.

The role of heme for *P. gingivalis* harbored in the oral cavity is considered to be protection from periodontal disease and maintenance of human health. As suggested in the supplementary information of this report, passive immunization may be a useful approach in the dental clinic. Unlike prevention of life-threatening diseases, use of a vaccine to elicit an immune response for a nonlife-threatening disease is not ethically acceptable, because of possible unexpected adverse effects (3). Therefore, passive immunization approaches against oral infectious

diseases have been pursued. Concerning human monoclonal antibodies against *P. gingivalis* cell surface proteins, such as rHBP35, passive immunization studies are feasible, though limitations include the empirical nature of this approach, as well as the inability to test its validity by infection of host gingival tissues with *P. gingivalis*.

Acknowledgements

This research was supported in part by 'Academic Frontier' Project for Private Universities, matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), a Grants-in-Aid for Scientific Research (C) (Kakenhi: 21390497, 22592078), and a Grant-in-Aid for Young Researchers of Nihon University School of Dentistry at Matsudo (2010-015).

References

1. Maiden MFJ, Carman RJ, Curtis MA *et al*. Detection of high-risk groups and individuals for periodontal diseases: laboratory markers based on the microbiological analysis of subgingival plaque. *J Clin Periodontol* 1990;**17:**1–13.
2. Slots J, Listgarten MA. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J Clin Periodontol* 1988;**15:**85–93.
3. Abiko Y. Passive immunization against dental caries and periodontal disease: development of recombinant and human monoclonal antibodies. *Crit Rev Oral Biol Med* 2000;**11:**140–158.
4. Abiko Y, Ogura N, Matsuda U, Yanagi K, Takiguchi H. A human monoclonal antibody which inhibits the coaggregation activity of Porphyromonas gingivalis. *Infect Immun* 1997;**65:**3966–3969.
5. Hosogi Y, Hayakawa M, Abiko Y. Monoclonal antibody against *Porphyromonas gingivalis* hemagglutinin inhibits hemolytic activity. *Eur J Oral Sci* 2001; **109:**109–113.
6. Abiko Y, Hayakawa M, Aoki H, Kikuchi T, Shimatake H, Takiguchi H. Cloning of a bacteroides-gingivalis outer-membrane protein gene in escherichia-coli. *Arch Oral Biol* 1990;**35:**689–695.
7. Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H. Role of *Porphyromonas gingivalis* 40-kDa outer-membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. *Arch Oral Biol* 1992;**37:**717–724.
8. Kawamoto Y, Hayakawa M, Abiko Y. Purification and immunochemical characterization of a recombinant outer-membrane protein from *Bacteroides gingivalis*. *Int J Biochem* 1991;**23:**1053–1061.
9. Shibata Y, Hiratsuka K, Hayakawa M *et al*. A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. *Biochem Biophys Res Commun* 2003;**300:**351–356.
10. Carvalho AP, Fernandes PA, Ramos MJ. Similarities and differences in the thioredoxin superfamily. *Prog Biophys Mol Biol* 2006;**91:**229–248.
11. Kadokura H, Katzen F, Beckwith J. Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem* 2003;**72:**111–135.
12. Pan JL, Bardwell JCA. The origami of thioredoxin-like folds. *Protein Sci* 2006;**15:**2217–2227.
13. Shiroza T, Okano S, Shibata Y *et al*. Functional analysis of the thioredoxin domain in *Porphyromonas gingivalis* HBP35. *Biosci Biotechnol Biochem* 2008;**72:**1826–1835.
14. Tomizuka K, Shinohara T, Yoshida H *et al*. Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. *Proc Natl Acad Sci U S A* 2000;**97:**722–727.
15. Takauchi A, Kobayashi T, Tahara T *et al*. The trans-chromosomic mouse-derived human monoclonal antibody promotes phagocytosis of *Porphyromonas gingivalis* by neutrophils. *J Periodontol* 2005;**76:**680–685.
16. Hamada N, Watanabe K, Tahara T *et al*. The r40-kDa outer membrane protein human monoclonal antibody protects against *Porphyromonas gingivalis*-induced bone loss in rats. *J Periodontol* 2007;**78:**933–939.
17. Hayakawa M, Hosogi Y, Takiguchi H *et al*. Evaluation of the electroosmotic medium pump system for preparative disk gel electrophoresis. *Anal Biochem* 2001;**288:**168–175.
18. Okano S, Shibata Y, Shiroza T, Abiko Y. Proteomics based analysis of a counter-oxidative stress system in *Porphyromonas gingivalis*. *Proteomics* 2006;**6:**251–258.
19. Kobayashi T, van der Pol WL, van de Winkel JGJ *et al*. Relevance of IgG receptor IIIb (CD16) polymorphism to handling of *Porphyromonas gingivalis*: implications for the pathogenesis of adult periodontitis. *J Periodont Res* 2000;**35:**65–73.
20. Bramanti TE, Holt SC. Roles of porphyrins and host iron transport proteins in regulation of growth of Porphyromonas-gingivalis W50. *J Bacteriol* 1991;**173:**7330–7339.
21. Marsh PD, McDermid AS, McKee AS, Baskerville A. The effect of growth-rate and hemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. *Microbiology UK* 1994;**140:**861–865.
22. Schifferle RE, Shostad SA, BayersThering MT, Dyer DW, Neiders ME. Effect of protoporphyrin IX limitation on *Porphyromonas gingivalis*. *J Endod* 1996;**22:** 352–355.
23. Amano A, Tamagawa H, Shizukuishi S, Tsunemitsu A. Superoxide dismutase, catalase and peroxidases in oral anaerobic bacteria. *J Osaka Univ Dent Sch* 1986;**26:**187–192.
24. Nakayama K. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. *J Bacteriol* 1994;**176:**1939–1943.
25. Shoji M, Shibata Y, Shiroza T *et al*. Characterization of hemin-binding protein 35 (HBP35) in *Porphyromonas gingivalis*: its cellular distribution, thioredoxin activity and role in heme utilization. *BMC Microbiol* 2010;**10:**152.
26. Marquis RE. Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J Ind Microbiol* 1995;**15:**198–207.
27. Diaz PI, Rogers AH. The effect of oxygen on the growth and physiology of *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 2004;**19:**88–94.