Oral Microbiology Immunology 2009: 24: 510–513
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Short communication

Influence of mouse prolactin-inducible protein in saliva on the aggregation of oral bacteria

A. Nistor[1], G. Bowden[2], A. Blanchard[1,3], Y. Myal[1,3]
[1]Departments of Pathology, [2]Oral Biology and [3]Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, MB, Canada

Nistor A, Bowden G, Blanchard A, Myal Y. Influence of mouse prolactin-inducible protein in saliva on the aggregation of oral bacteria.
Oral Microbiol Immunol 2009: 24: 510–513. © 2009 John Wiley & Sons A/S.

Introduction: Mouse prolactin-inducible protein (mPIP) is secreted in mouse saliva and has been found to bind oral bacteria, showing the highest affinity for streptococci. Comparisons between the oral flora of mPIP knockout mice and their wild-type controls showed differences in the genera colonizing the two groups of mice. These findings suggested a role for mPIP in the colonization of the mouse oral cavity, possibly modulating the oral flora. In this *in vitro* study, we focused on the contribution of this protein to aggregation of oral bacteria, a process thought to promote the clearance of bacteria from the oral cavity, and one that could influence the composition of the oral bacterial community.
Methods: The aggregation of selected human and mouse oral streptococci was measured spectrophotometrically. The aggregation of oral bacteria by saliva from mPIP knockout mice, which lack mPIP, was compared with that of saliva from wild-type mice.
Results: Both wild-type saliva and mPIP knockout mouse saliva induced aggregation of human strain *Streptococcus gordonii* SK120 and mouse streptococci strains M105/6 and M106/2. Bacterial aggregation induced by the saliva of wild-type mice was significantly higher than the aggregation induced by saliva from mPIP knockout mice for all the bacterial strains.
Conclusion: In this study it was confirmed that mPIP plays a role in the aggregation of oral bacteria. The salivary components promoting aggregation of oral bacteria are considered to be part of the oral defense mechanisms so these findings provide insight into a possible function of mPIP in host defense by promoting aggregation of oral bacteria.

Key words: aggregation; bacteria; GCDFP-15; knockout mice; prolactin-inducible protein; saliva; streptococci

Andreea Nistor, 727 McDermot Avenue, Winnipeg, MB, Canada R3E 3P5
Tel.: +1 204 789 3808;
fax: +1 204 789 3931;
e-mail: Andreea.nistor@sympatico.ca

Accepted for publication June 25, 2009

The mouse prolactin-inducible protein/mouse submaxillary gland protein (mPIP/mSMGP) is the murine homologue of human prolactin-inducible protein/gross cystic disease fluid protein-15 (PIP/GCDFP-15), an established marker for both malignant and benign pathological conditions of the human breast. As with human PIP, mPIP messenger RNA is also abundant in the submaxillary gland (24, 30), its expression being limited to the lacrimal and major salivary glands of the mouse (17, 24–26). Murine PIP was found in mouse lacrimal, submaxillary, and parotid glands and was localized as a diffuse cytoplasmic immunohistochemical staining of serous acinar cells of both lacrimal and submaxillary glands of the mouse (25). In addition, secreted mPIP has been identified in mouse saliva and tears (5, 16, 25).

While a function for human PIP has not yet been defined, several findings suggest a potential role for this protein in immunomodulation and host defense mechanisms (6, 8, 13). It was also proposed that as a CD4-binding factor (1, 2, 10), PIP may provide protection from infection with human immunodeficiency virus (HIV) by interfering with the HIV envelope/CD4 binding (3). Human PIP has also been shown to possess aspartyl protease activity, specifically degrading fibronectin (7). In addition, human PIP was found to bind to several human oral and non-oral bacterial species, including oral streptococci (28, 29).

As with its human counterpart, the exact functions of mPIP are not known. Through *in vitro* studies, we demonstrated that mPIP binds to several strains of both human and mouse oral bacteria, showing the highest affinity for the genus *Streptococcus* (16). Although the consequences of this binding are not known, we suggest that mPIP might modulate the resident oral flora of mice (16). We further demonstrated both quantitative and qualitative differences between the oral flora of mPIP knockout mice that lack mPIP and that of wild-type mice (25).

Salivary components may impact on bacterial colonization of the oral cavity by interacting with oral bacteria in different ways, including binding to microorganisms, resulting in their aggregation and facilitating their clearance from the oral cavity; serving as receptors for microbial adhesion to host surfaces; possessing antibacterial activity; or serving as microbial nutritional substrates (27). Bacterial aggregation is a process thought to promote the clearance of microorganisms from the oral cavity (22) and salivary components that promote this phenomenon are considered components of oral defense systems (9).

The aim of the present study was to further investigate the interaction between mPIP and oral bacteria; specifically, to determine whether mPIP plays a role in the aggregation of oral bacteria and thereby playing a role in non-immune host defense.

Materials and methods

Knockout mice lacking mPIP ($mPIP^{-/-}$) were generated by gene targeting through homologous recombination (5, 25). The mPIP null mutation was generated by excluding exon 2 from the construct and replacing it with a PGK-neo cassette. Negative selection was carried out through use of a diphtheria toxin A cassette (PGK-DTA) inserted on the 3′ end of the construct. The 13.1-kb linearized PIP targeting vector was outsourced to the Gene Targeting & Transgenic Facility, University of Connecticut Health Center, Farmington, CT, for the generation of the knockout mice. Briefly, the targeting vector was electroporated into 129/Svj embryonic stem cells. Embryonic stem cells generated from three confirmed mPIP-targeted clones were injected into 3-day-old C57BL/6 blastocysts, which were implanted into pseudo-pregnant female recipients. Mice positive for the mPIP null gene in the germline were shipped to the University of Manitoba Central Animal Care Facility where they were backcrossed into a CD1 genetic background.

Saliva was collected from both wild-type and mPIP knockout adult mice as previously described (16, 25). Western blot analysis was used to confirm the absence of mPIP in saliva collected from mPIP knockout mice (5, 25).

Human *Streptococcus gordonii* SK120 strain isolated from the human oral cavity (12) was generously donated by Dr Mogens Kilian (University of Aarhus, Denmark). Streptococcal strains M105/6 and M106/2 were isolated from the mouse oral cavity and characterized by us (25). Bacteria were cultured for 16–24 h in a candle jar on blood agar plates (Blood agar base no. 2; Oxoid, Hampshire, UK), supplemented with 1% laked horse blood (Oxoid). Colonies were used to inoculate 40 ml Todd–Hewitt broth; they were grown for 16 h in a candle jar and harvested by centrifugation at 10,000 ***g*** for 10 min.

Bacterial pellets were washed twice and resuspended in phosphate-buffered saline (PBS) to an optical density at 700 nm (OD_{700}) of 1 (± 0.02). Bacterial aggregation was measured using a modification of the method of Ericson et al. (9). The aggregation reactions were made by mixing bacterial suspensions with 100–200 μl pooled mouse saliva collected from either $mPIP^{-/-}$ or wild-type (WT) mice to a final volume of 1 ml. The PBS was used as a control for autoaggregation. The reaction mixtures were placed into cuvettes and incubated at room temperature without further mixing. The OD_{700} of the mixtures was measured with a spectrophotometer at set time intervals (10–20 min) for a maximum of 2 h. A decrease in the OD_{700} of the mixture, accompanied by the visualization of macroscopic aggregates floating or settled at the bottom of the cuvettes at the end of the 2-h period, was considered positive for saliva-induced aggregation.

Bacterial aggregation was recorded as a decrease of the OD_{700} of the mixture of bacterial suspension and mouse saliva, expressed as percentage of the optical density of the original mixture. The values determined at each time-point were plotted against time on an *xy* graph, the starting point representing time 0 and 100% optical density. For each experiment, aggregation assays were set up in duplicate and the data obtained from each set of duplicates were averaged. The level of aggregation induced by saliva derived from WT mice was compared with that of $mPIP^{-/-}$ mouse saliva.

Results

Both WT and $mPIP^{-/-}$ mouse saliva were able to aggregate the bacterial strains tested. No autoaggregation was detected in the controls where PBS was used instead of saliva. After 2 h of incubation of bacteria with mouse saliva the level of aggregation induced by the WT mouse saliva was higher than that induced by $mPIP^{-/-}$ mouse saliva, a finding that was consistent for both human strain SK120 and mouse strains M105/6 and M106/2 (Fig. 1).

Among the three strains, the mean difference in OD_{700} between the WT and the PBS control was 25.5 ± 2.1, whereas the mean difference in OD_{700} between the $mPIP^{-/-}$ and the PBS control was 13.3 ± 0.7 (two-tailed $P < 0.01$, Student's unpaired *t*-test).

Discussion

Considered to be a process that promotes the clearance of bacteria from the oral cavity (22), bacterial aggregation has been widely studied and different assays have been developed to determine the aggregation of oral bacteria by human saliva (15). In addition to aggregation of bacteria by human whole saliva, the effect of different components of human saliva on the aggregation of oral bacteria has also been explored (4, 18, 19, 31). However, less is known about the effects of the saliva of other species that are often used in laboratory animal models, such as mice, on bacterial aggregation.

Strains of *Streptococcus* were used in this study because *Streptococcu*s species are among the commonest bacteria in the mouth and comprise a significant component of human dental plaque (23). Human *S. gordonii* (SK120) was selected as a positive control when testing mouse saliva for aggregation because it has been shown to bind human PIP (our unpublished data). Also, bacterial aggregation can be strongly strain-dependent (14) and human saliva has been shown to aggregate this strain (20). Mouse streptococci strains M105/6 and M106/2 were randomly selected from a large number of mouse isolates (25). In this study the two mouse *Streptococcus* strains were only classified to the level of genus and, unfortunately, it was not possible to undertake an extensive examination of their biological and physiological characteristics. Identification of oral streptococci from mice to species level is often difficult because of the lack of taxonomic data for mouse isolates. However, in other

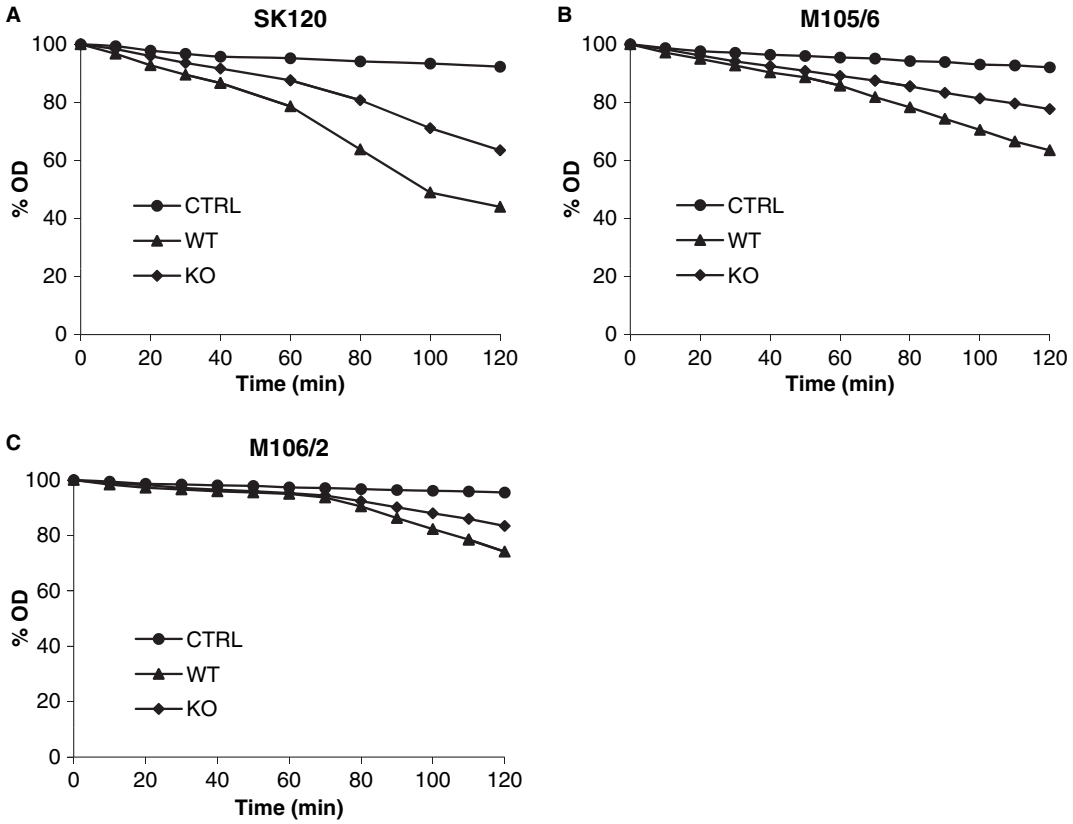

Fig. 1. Bacterial aggregation assays. Saliva from both wild-type (WT) and mPIP$^{-/-}$ (knockout; KO) mice induced aggregation of the human *Streptococcus gordonii* strain SK120 (A) and of mouse oral streptococci strains M105/6 (B) and M106/2 (C). For all three strains, WT saliva induced a higher degree of aggregation than mPIP$^{-/-}$ saliva. No autoaggregation was observed in the phosphate-buffered saline controls (CTRL).

studies (16) we have described mouse strains in terms of their close similarity to well-defined human species.

Previous work from our laboratory (16) has demonstrated that mPIP binds to human and mouse oral bacteria with the highest affinity for streptococci. However, the consequences of this interaction were not known. Suggested hypotheses were that mPIP caused or facilitated bacterial aggregation, mediated adhesion to oral tissues, or caused bacterial lysis, playing a role in innate host defense. This is particularly important because the resident oral flora benefits the host by providing protection against colonization by pathogenic organisms (21).

The significant reduction in aggregation of bacteria by saliva from mPIP$^{-/-}$ compared with WT mice may be attributed to the absence of PIP from the saliva of mPIP$^{-/-}$ mice. However, the role of mPIP in the aggregation of bacteria by mouse saliva has not been defined and saliva contains other carbohydrates and proteins that bind to oral streptococci and may also promote aggregation (18–20, 27, 31). Murine PIP could play a direct role or an ancillary role to these molecules. Our attempts to produce active recombinant mPIP and to isolate the protein from mouse saliva using antibody-affinity columns to confirm the direct involvement of mPIP in the aggregation of oral bacteria were unsuccessful (25). Similarly, difficulties in purifying human PIP from body fluids were encountered by others, demonstrating that it is a difficult task; this can be attributed to the tendency of PIP to aggregate and form multimers or complexes with other protein species (1, 11). Also, we cannot be sure that the deletion of mPIP in the mouse does not influence the activities or expression of other molecules in mouse saliva that aggregate oral bacteria *in vitro* or *in vivo*. As an example, fibronectin present in human saliva can cause the aggregation of human oral streptococci (30). PIP may degrade fibronectin (7) and consequently influence any fibronectin-based aggregation. However, we can say that independent of a known aggregation mechanism, removal of mPIP from mouse saliva causes a reduction in saliva-induced bacterial aggregation *in vivo*, suggesting that mPIP plays a role in any process or cascade causing aggregation of mouse oral streptococci by mouse saliva.

In this study we examined the differences between the levels of aggregation induced by mPIP knockout mouse saliva, which lacks mPIP, and wild-type mouse saliva. We demonstrated that mPIP contributes to saliva-induced bacterial aggregation and we suggest that mPIP might play a role in host defense by modulating the resident oral flora by facilitating bacterial aggregation.

Acknowledgments

We would like to thank Dr Mogens Kilian (University of Aarhus, Denmark) for donating the SK120 bacterial strain used in this study. Andreea Nistor was a recipient of a Manitoba Graduate Scholarship and a Health Sciences Centre Foundation Graduate Studentship. Yvonne Myal received a Manitoba Medical Services Foundation Career Award. This study was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC).

References

1. Autiero M, Abrescia P, Guardiola J. Interaction of seminal plasma proteins with cell surface antigens: presence of a CD4-binding glycoprotein in human seminal plasma. Exp Cell Res 1991: **197**: 268–271.
2. Autiero M, Cammarota G, Friedlein A et al. A 17-kDa CD4-binding glycoprotein present in human seminal plasma and in breast tumor cells. Eur J Immunol 1995: **25**: 1461–1464.
3. Autiero M, Gaubin M, Mani JC et al. Surface plasmon resonance analysis of gp17, a natural CD4 ligand from human seminal plasma inhibiting human immunodeficiency virus type-1 gp120-mediated syncytium formation. Eur J Biochem 1997: **245**: 208–213.
4. Babu JP, Dabbous MK. Interaction of salivary fibronectin with oral streptococci. J Dent Res 1986: **65**: 1094–1100.
5. Blanchard AAA, Nistor A, Castaneda FE, Martin D, Hicks GG, Amara F, Shiu RPC, Myal Y. Generation and initial characterization of the prolactin inducible protein (PIP) null mice: accompanying global changes in gene expression in the submandibular gland. Can J Physiol Pharmacol 2009: **87**: in press.
6. Bryborn M, Adner M, Cardell LO. Psoriasin, one of several new proteins identified in nasal lavage fluid from allergic and non-allergic individuals using 2-dimensional gel electrophoresis and mass spectrometry. Respir Res 2005: **6**: 118.
7. Caputo E, Manco G, Mandrich L, Guardiola J. A novel aspartyl proteinase from apocrine epithelia and breast tumors. J Biol Chem 2000: **275**: 7–935.
8. Chiu WW, Chamley LW. Human seminal plasma prolactin-inducible protein is an immunoglobulin G-binding protein. J Reprod Immunol 2003: **60**: 97–111.
9. Ericson T, Pruitt K, Wedel H. The reaction of salivary substances with bacteria. J Oral Pathol 1975: **4**: 307–323.
10. Gaubin M, Autiero M, Basmaciogullari S et al. Potent inhibition of CD4/TCR-mediated T cell apoptosis by a CD4-binding glycoprotein secreted from breast tumor and seminal vesicle cells. J Immunol 1999: **162**: 2631–2638.
11. Haagensen DE Jr, Mazoujian G, Dilley WG et al. Isolation and radioimmunoassay for a major component protein. J Natl Cancer Inst 1979: **62**: 239–247.
12. Kilian M, Mikkelsen L, Henrichsen J. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp.nov. and emended descriptions of *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus mitis*. Int J Syst Bacteriol 1989: **39**: 471–484.
13. Koo BS, Lee DY, Ha HS, Kim JC, Kim CW. Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis. J Proteome Res 2005: **4**: 719–724.
14. Koop HM, Valentijn-Benz M, Nieuw Amerongen AV, Roukema PA, de Graaff J. Aggregation of 27 oral bacteria by human whole saliva. Influence of culture medium, calcium, and bacterial cell concentration, and interference by autoaggregation. Antonie Van Leeuwenhoek 1989: **55**: 277–290.
15. Koop HM, Valentijn-Benz M, Nieuw Amerongen AV, Roukema PA, de Graaff J. Comparison of different assays for the aggregation of oral bacteria by human whole saliva. Antonie Van Leeuwenhoek 1989: **55**: 109–122.
16. Lee B, Bowden GH, Myal Y. Identification of mouse submaxillary gland protein in mouse saliva and its binding to mouse oral bacteria. Arch Oral Biol 2002: **47**: 327–332.
17. Lee B, Modha G, Watson PH et al. Expression of the mouse homologue for the human GCDFP-15/PIP gene during pre- and early post-natal development. Mol Cell Endocrinol 2003: **205**: 33–41.
18. Ligtenberg AJ, Veerman EC, de Graaff J, Nieuw Amerongen AV. Influence of the blood group reactive substances in saliva on the aggregation of *Streptococcus rattus*. Antonie Van Leeuwenhoek 1990: **57**: 97–107.
19. Ligtenberg AJ, Veerman EC, de Graaff J, Nieuw Amerongen AV. Saliva-induced aggregation of oral streptococci and the influence of blood group reactive substances. Arch Oral Biol 1990: **35** (suppl): 141S–143S.
20. Ligtenberg AJ, Walgreen-Weterings E, Veerman EC, de Soet JJ, de Graaff J, Amerongen AV. Influence of saliva on aggregation and adherence of *Streptococcus gordonii* HG 222. Infect Immun 1992: **60**: 3878–3884.
21. Liljemark WF, Bloomquist C. Human oral microbial ecology and dental caries and periodontal diseases. Crit Rev Oral Biol Med 1996: **7**: 180–198.
22. Mandel ID. In defense of the oral cavity. In: Kleinberg I, Ellison SA, Mandel ID ed. Saliva and dental caries. New York, NY: Information Retrieval Inc, 1979: 473–491.
23. Marsh P, Martin MV. Oral microbiology, 4th edn. Oxford, UK: Butterworth-Heinemann, 1999.
24. Myal Y, Iwasiow B, Yarmill A, Harrison E, Paterson JA, Shiu RP. Tissue-specific androgen-inhibited gene expression of a submaxillary gland protein, a rodent homolog of the human prolactin-inducible protein/GCDFP-15 gene. Endocrinology 1994: **135**: 1605–1610.
25. Nistor A. Investigations into the role of mPIP, the mouse homologue of hPIP/GCDFP-15, in innate host defense. Winnipeg, MB: MSc University of Manitoba, 2008. http://hdl.handle.net/1993/3031
26. Osawa M, Horiuchi H, Tian W, Kaneko M. Divergent evolution of the prolactin-inducible protein gene and related genes in the mouse genome. Gene 2004: **325**: 179–186.
27. Scannapieco FA. Saliva–bacterium interactions in oral microbial ecology. Crit Rev Oral Biol Med 1994: **5**: 203–248.
28. Schenkels LC, Ligtenberg AJ, Veerman EC, Van Nieuw AA. Interaction of the salivary glycoprotein EP-GP with the bacterium *Streptococcus salivarius* HB. J Dent Res 1993: **72**: 1559–1565.
29. Schenkels LC, Walgreen-Weterings E, Oomen LC, Bolscher JG, Veerman EC, Nieuw Amerongen AV. *In vivo* binding of the salivary glycoprotein EP-GP (identical to GCDFP-15) to oral and non-oral bacteria detection and identification of EP-GP binding species. Biol Chem 1997: **378**: 83–88.
30. Windass JD, Mullins JJ, Beecroft LJ et al. Molecular cloning of cDNAs from androgen-independent mRNA species of DBA/2 mouse sub-maxillary glands. Nucleic Acids Res 1984: **12**: 1361–1376.
31. Yamaguchi T. Human salivary aggregation in *Streptococcus intermedius* type g strains: relationship with IgA. FEMS Immunol Med Microbiol 2004: **41**: 101–107.