Protease-Activated Receptor-2 Activation

A Major Role in the Pathogenesis of Porphyromonas gingivalis Infection

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We have investigated the specific contribution of protease-activated receptor-2 (PAR₂) to host defense during *Porphyromonas gingivalis* infection. Culture supernatants from *P. gingivalis* strains 33277 and W50 provoked Ca²⁺ mobilization in cells transfected with PAR₂ (PAR₂-KNRK) and desensitized the subsequent responses to PAR₂-selective agonist. In addition, culture supernatants of *P. gingivalis* E8 (RgpA/RgpB double knockout) did not cause calcium response in PAR₂-KNRK cells, evidencing the involvement of the arginine-specific cysteine proteases RgpA and RgpB in PAR₂ activation by *P. gingivalis*. Injection of *P. gingivalis* into mouse subcutaneous chambers provoked an increased proteolytic activity, which was inhibited by serine protease inhibitors. Fluids collected from chambers of *P. gingivalis*-injected mice were able to activate PAR₂ and this activation was inhibited by serine protease inhibitors. *P. gingivalis* inoculation into subcutaneous chambers of wild-type mice induced an inflammatory response that was inhibited by a serine protease inhibitor and was significantly reduced in PAR₂-deficient mice. Finally, mice orally challenged with *P. gingivalis* developed alveo-

lar bone loss, which was significantly reduced in PAR₂-deficient mice at 42 and 60 days after *P. gingivalis* infection. We conclude that PAR₂ is activated on *P. gingivalis* infection, in which it plays an important role in the host inflammatory response. (*Am J Pathol* 2006, 168:1189–1199; DOI: 10.2353/ajpath.2006.050658)

The protease-activated receptors (PARs) belong to a family of G-protein-coupled, seven-transmembrane-domain receptors.^{1,2} Activation of PARs occurs through proteolytic cleavage of the extracellular domain, resulting in generation of a new N-terminal tethered ligand.³ To date, four PARs have been identified: PAR₁, PAR₂, PAR₃, and PAR₄.^{4,5} For PAR₁, PAR₂, and PAR₄, synthetic peptide agonists corresponding to the newly created N-terminus are able to activate the receptor in the absence of receptor cleavage.^{6,7}

Although these receptors have a similar mechanism of activation,⁸ they may have different biological functions and tissue distribution, and they can be activated by different proteases. PAR₁, PAR₃, and PAR₄ are activated by thrombin and are implicated in platelet aggregation.⁸ Trypsin, mast cell tryptase, neutrophil protease 3, tissue factor/factor VIIa/factor Xa, membrane-tethered serine protease-1, and gingipains have been identified as activators of PAR₂.^{9–11} Some studies have shown that PAR₂ participates in inflammatory processes *in vivo*.^{12,13} Although PAR₂ activation causes edema and granulocyte

Supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Foundation, the Canadian Institute of Health Research (MPO-22362 to N.V. and MPO-5619 to R.P.E.), the Alberta Heritage Foundation for Medical Research (to N.V.), and Interdisziplinäres Zentrum für Klinische Forschung Leipzig grants (SFB492,293), Serono, Centre de Recherches et d'Investigations Épidémiologiques et Sensorielles (to M.S.).

Accepted for publication December 16, 2005.

N.V. is an Alberta Heritage Foundation for Medical Research Scholar and a Canadian Institute of Health Research new investigator.

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recruitment in the rat paw,¹³ the role of PAR₂ on mucosal surfaces is not as clearly defined. PAR₂ agonists exert protective effects in airways against lipopolysaccharide challenge¹⁴ and in the colon in a model of chronic colitis induced by trinitrobenzene sulfonic acid,¹⁵ but they are proinflammatory when acutely administered in the colon¹⁶ or in the airways¹⁷ of mice.

Recently, some studies have suggested a role for PAR₂ in periodontitis, a chronic oral inflammation leading to bone and tooth loss, because it was found to be expressed by osteoblasts, oral epithelial cells, and human gingival fibroblasts.^{9,11,18} Importantly, Loubakos and colleagues⁹ reported that bacterial cysteine proteases such as Arg-gingipain (Rgp), which are produced by *Porphyromonas gingivalis* (a major causative agent of chronic periodontitis), was able to activate PAR₂ in oral epithelial cells and to induce the secretion of the proinflammatory cytokine interleukin (IL)-6, which is a potent stimulator of osteoclast differentiation and bone resorption. The production of potent proinflammatory mediators was also shown by Uehara and colleagues¹¹ who demonstrated that a synthetic PAR₂ agonist peptide activated the production of IL-8 in human gingival fibroblasts. In a previous study,¹⁹ we have evaluated the *in vivo* effects of PAR₂ activation by a selective agonist (SLIGRL-NH₂) on periodontal disease in rats. The PAR₂ agonist caused periodontitis (inflammation and alveolar bone loss) through a mechanism involving prostaglandin release and matrix metalloproteinase activation. Taken together, these studies strongly suggest a role for PAR₂ activation in inducing inflammation and bone resorption during periodontitis. However, a study by Smith and colleagues²⁰ in 2004 suggested an opposite role for PAR₂ activation: they hypothesized that PAR₂ activation may inhibit bone resorption in the context of periodontal diseases. They showed that PAR₂ agonists inhibited osteoclast differentiation, therefore being possibly protective against bone loss signals. Interestingly, Chung and colleagues²¹ have identified PAR₂ as a receptor used by Rgp in induction of human β -defensin-2 (hBD-2) in oral epithelial cells and have also demonstrated hBD-2 induction by PAR₂ peptide agonist. Notably, antimicrobial peptides of the hBD family are part of the innate immune responses that play a role in mucosal defense. No definitive answer on the role of PAR₂ in oral mucosal inflammation or in periodontal diseases (pro- or anti-inflammatory effect, pro- or anti-bone loss signal) has so far emerged. Therefore, our strategy was to use a genetic approach, using PAR₂-deficient mice, to study the specific contribution of PAR₂ activation and proteolytic activity during infection by the proteolytic periodontal pathogen *P. gingivalis*.

Materials and Methods

Animals

PAR₂-deficient (PAR₂^{-/-}) and wild-type (WT) littermate (PAR₂^{+/+}) mice, both of the C57BL6 background²² originally obtained from Johnson and Johnson Pharmaceutical Research Institute (Spring House, PA), were bred at

the University of Calgary animal care facility. Six- to nine-week-old mice were used in the present study. All animals were housed in a temperature-controlled room; food and water were provided *ad libitum*. The Animal Care and Ethics Committees of the University of Calgary approved all experimental protocols, which followed the guidelines of the Canadian Council on Animal Care.

Bacteria

P. gingivalis strains 33277 [American Type Culture Collection (ATCC), Rockville, MD], W50 (WT), and E8 (RgpA/RgpB double mutant, obtained from Dr. J. Aduse-Opoku, Queen Mary's School of Medicine and Dentistry, London, UK) were grown on anaerobic blood agar plates in an anaerobic chamber with 85% N₂, 5% H₂, and 10% CO₂. After incubation at 37°C for 7 days, the bacteria were collected and suspended in Schaedler broth (Difco Laboratories, Detroit, MI) to a final optical density of 1.2 (10⁹ CFU/ml) at 660 nm. *Treponema denticola* strain ATCC 35405 was obtained from the culture collection at the University of Toronto. It was grown in modified new oral spirochete medium²³ for 3 days (mid-exponential phase) to a concentration of 2.8 × 10⁹ cells/ml, as determined by microscopic count.

Subcutaneous Chamber

Coil-shaped chambers were prepared from 0.5-mm stainless-steel wire and were surgically implanted in the subcutaneous tissue of the dorso-lumbar region of each mouse, as previously described.²⁴ Ten days after implantation, chambers were inoculated with 0.1 ml of *P. gingivalis* 33277, *P. gingivalis* W50, or *P. gingivalis* E8, suspended in sterile saline (10⁹ cells/ml). Control mice were inoculated with vehicle only. Mice were sacrificed by cervical dislocation at 1 day after bacterial inoculation. Another group of mice was inoculated with 0.1 ml of a solution containing *P. gingivalis* (10⁹ cells/ml) and 0.3 mg/ml of SBTI (soybean trypsin inhibitor; Sigma, St. Louis, MO) and sacrificed 1 day after.

Subcutaneous Chamber Fluid Analysis

A sample of chamber fluid (100 μ l) was aseptically collected from each animal at 1 day after *P. gingivalis* or vehicle challenge to assess the host inflammatory response. A 10- μ l aliquot of each chamber fluid was diluted 10-fold in Turk's staining solution to determine the total number of inflammatory cells by light microscopy, using a Neubauer counting chamber (Hauser Scientific, Horsham, PA). The rest of the samples were aliquoted and kept for evaluation of proteolytic activity as well as cytokine and prostaglandin release.

Cell Culture

PAR₂-expressing Kirsten sarcoma-transformed rat kidney epithelial cells (KNRK: ATCC, Manassas, VA) were

propagated in geneticin (0.6 mg/ml)-containing medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% Penstrep) to maintain vector-selective pressure. Nontransfected KNRK cells were grown in geneticin-free medium. Cells at 90% confluence in 80-cm² flasks (Life Technologies, Inc., Grand Island, NY) were rinsed with phosphate-buffered saline, lifted with nonenzymatic cell dissociation fluid, and pelleted before resuspension in 1 ml of Hanks' balanced salt solution (pH 7.4), 2.5 μ l of sulfinpyrazone (100 mmol/L), 1 μ l of a 20% Pluronic F-127 solution, and 10 μ l of 2.5 mg/ml Fluo-3 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR). The final solution was incubated at room temperature while shaking gently for 25 minutes. Cells were then washed three times and resuspended in calcium assay buffer (150 mmol/L NaCl, 3 mmol/L KCl, 1.5 mmol/L CaCl₂, 10 mmol/L glucose, 20 mmol/L HEPES, 0.25 sulfinpyrazone, pH 7.4).

Calcium Signaling Assay

Determination of intracellular calcium mobilization was performed as previously described.²⁵ Briefly, fluorescence measurements were performed on a fluorescence spectrometer 650-10S (Perkin-Elmer, Norwalk, CT). Suspensions of cells (5×10^5 cells/ml) loaded with the calcium fluorochrome fluo-3 (Molecular Probes, Inc.) were placed in 4-ml cuvettes, stirred with a magnetic flea bar, and maintained at 24°C.

The signal produced by PAR₂-expressing KNRK or nontransfected KNRK cells was measured after the addition of culture supernatants (40 μ l) of *P. gingivalis* 33277, W50, or E8, and *T. denticola* 35405. In addition, we also evaluated the signal produced by PAR₂-expressing KNRK with the addition of supernatants from chamber fluid samples, previously incubated with either 10 nmol/L SBTI (trypsin inhibitor), 1 mmol/L PMSF (phenylmethyl sulfonyl fluoride) (serine proteinase inhibitor, Sigma), 1 mmol/L TLCK (*N*-*p*-tosyl-L-lysine chloromethyl ketone, cysteine proteinase inhibitor; Sigma), or 1 mmol/L leupeptin (Rgp cysteine proteinase inhibitor, Sigma) for 10 minutes. The calcium mobilization after the addition of culture supernatants of *P. gingivalis* 33277, W50, or E8 preincubated with either 1 mmol/L TLCK or 1 mmol/L leupeptin for 10 minutes was evaluated. The calcium responses to known PAR₂ agonists [2 nmol/L trypsin (Sigma) or 2 μ mol/L of SLIGRL-NH₂] were also evaluated. The results are expressed as percentages (% A23187) of the fluorescence emission (E530) caused by 2- μ mol/L concentrations of the calcium ionophore A23187. The PAR₂ agonist peptide was synthesized by the Peptide Synthesis Facility (University of Calgary, Calgary, AB, Canada). All experiments were repeated three to five times, for at least eight samples.

Proteolytic Activity

After centrifugation of the samples from the fluid chambers, supernatants were collected and assayed for proteolytic activity using a Fluoroskan Ascent fluorimeter

(Thermo Electron, Franklin, MA). Besides testing the proteolytic activity of the supernatants of chamber fluid samples, we also evaluated the effect of preincubation (10 minutes) of the samples with either 10 nmol/L SBTI (trypsin inhibitor), 1 mmol/L PMSF (serine proteinase inhibitor, Sigma), 1 mmol/L TLCK (serine and cysteine proteinase inhibitor, Sigma), or 1 mmol/L leupeptin (serine and Rgp cysteine proteinase inhibitor, Sigma). Assays were performed at 25°C, with a 355-nm excitation wavelength filter and a 460-nm emission wavelength filter. Fluorescence from wells on the microplate was measured throughout 20 minutes. The hydrolysis of the substrate (5 μ l) added to sample solution (75 μ mol/L Boc-Gln-Ala-Arg-AMC in 50 mmol/L Tris/20 mmol/L CaCl₂ buffer, pH 7.4) was calibrated with the rate of AMC hydrolysis product in a standard reaction mixture using a serial dilution of trypsin (0.05 to 2 U/ml). All assays were performed in triplicate; the range of values observed was always less than 10% of the mean. The results are expressed in units of trypsin per milliliter (U/ml).

Measurement of Prostaglandin-E₂, Interferon (IFN)- γ , IL-1, IL-6, and IL-10 Levels

Prostaglandin-E₂, IFN- γ , IL-1, IL-6, and IL-10 levels in the supernatants of chamber fluid samples collected from mice that received inoculation of *P. gingivalis* 33277 or vehicle were determined by using commercially available enzyme-linked immunosorbent assay kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN). The concentration of the inflammatory mediators was determined using the Softmax data analysis program (Molecular Devices, Menlo Park, CA).

Oral Infection

A total of 0.2 ml of *P. gingivalis* 33277 suspended in sterile saline (10^9 cells) was given to each mouse via a gavage needle every other day for a total of 4 days, in part by gavage, and by local application in the oral cavity. Mice were then allowed free access to standard mouse chow and water. The mice were sacrificed by cervical dislocation 42 and 60 days after the last bacterial administration. Ten sham-infected (sterile saline-injected) and 10 *P. gingivalis*-infected mice were used at each time point in each animal group (PAR₂^{-/-} or WT mice). Mandibles were removed, hemisected, exposed to NaOH (2N), and then mechanically defleshed.

Alveolar Bone Loss

Horizontal bone loss around the mandibular molars was assessed by measuring the distance between the cemento-enamel junction and alveolar bone crest under a dissecting microscope ($\times 40$). Measurements of bone level were done at seven sites on the lingual side of the left and right mandibular molars, and a total of 14 measurements per mouse were done three times in a random, blinded protocol by one evaluator.

Statistical Analysis

One-way analysis of variance was used to compare means among groups. In case of significant differences among the groups, posthoc two-group comparisons were assessed with Tukey-Kramer test. A *P* value <0.05 was considered statistically significant. Data are expressed as mean $\pm$ SE.

Results

Proteases Released by *P. gingivalis* Activate PAR₂

To evaluate the role of proteolytic activity produced by the bacteria itself, we examined the effects of *P. gingivalis* 33277, *P. gingivalis* W50, and *P. gingivalis* E8 culture supernatants on Ca²⁺ mobilization in PAR₂-KNRK cells (Figure 1). We first confirmed that in the KNRK cell line, no calcium mobilization was observed after addition of any of the *P. gingivalis* culture supernatants or trypsin (data not shown). Then, we showed that *P. gingivalis* culture supernatants (40 μ l) from strains 33277 and W50 provoked Ca²⁺ mobilization in PAR₂-KNRK cells. However, the calcium response induced by culture supernatants of *P. gingivalis* (33277 and W50) was significantly lower than the response to 2 μ mol/L of the selective agonist SLIGRL-NH₂ (Figure 1A). Preincubation of PAR₂-KNRK cells with culture supernatants of *P. gingivalis* (33277 and W50) significantly reduced the subsequent response of those cells to SLIGRL-NH₂ (Figure 1A). We demonstrated that after desensitization of PAR₂ receptor by two sequential treatments with trypsin for 5 minutes, the addition of *P. gingivalis* 33277 culture supernatant did not cause any calcium response in PAR₂-KNRK cells (Figure 1B, XI). Taken together, these results show that proteases released by *P. gingivalis* 33277 and *P. gingivalis* W50 activate PAR₂ in PAR₂-transfected cells.

The involvement of the cysteine proteases Arg-gingipain in PAR₂ activation was delineated by two different approaches. First we used the culture supernatants from strains *P. gingivalis* W50 (WT) and E8 (RgpA/RgpB double knockout). We found that *P. gingivalis* W50 culture supernatants (40 μ l) provoked Ca²⁺ mobilization in PAR₂-KNRK cells and decreased the subsequent responses to SLIGRL-NH₂ or trypsin (Figure 1B, III and VIII). No significant difference was found between the two strains of *P. gingivalis* (33277 and W50, Figure 1A). However, *P. gingivalis* E8 culture supernatant (40 μ l) did not cause calcium response in PAR₂-KNRK cells and did not alter the subsequent responses to trypsin or SLIGRL-NH₂ (Figure 1B, IV and IX; not shown for SLIGRL-NH₂). Then, we found that preincubation of *P. gingivalis* 33277 and *P. gingivalis* W50 culture supernatant with two serine/cysteine protease inhibitors, TLCK and leupeptin, led to a significant increase in the subsequent response to SLIGRL-NH₂ compared to untreated supernatants (Figure 1A). The *P. gingivalis* 33277 and *P. gingivalis* W50 culture supernatant-induced PAR₂ desensitization was inhibited by serine/cysteine protease inhibitors (leupeptin and

A.

B.

Figure 1. Calcium response in PAR₂-expressing KNRK cells to the addition of culture supernatant samples from *P. gingivalis* (P.g.) 33277, *P. gingivalis* W50, *P. gingivalis* E8, or *T. denticola* (T.d.). **A:** Effects of the PAR₂ agonist SLIGRL-NH₂ (2 μ mol/L) or of culture supernatants from *P. gingivalis* 33277 or *P. gingivalis* W50 on calcium mobilization in PAR₂-transfected KNRK cells. Some cells were preincubated with the culture supernatants of *P. gingivalis* 33277 or *P. gingivalis* W50 (shown at the top of the bars) in the presence or not of 1 mmol/L TLCK or 1 mmol/L leupeptin. *Significant difference versus calcium response generated by the same sample without protease inhibitor treatment. Increases in intracellular calcium were monitored as a percentage (% A23187) of the fluorescence emission (E530) caused by 2- μ mol/L concentrations of the calcium ionophore A23187. Means $\pm$ SEM from eight separate experiments. **B:** Schematic figures showing the calcium response in PAR₂-expressing cells (KNRK) to the addition of PAR₂ agonists SLIGRL-NH₂ (I), trypsin (VI), or culture supernatant samples from *P. gingivalis* 33277 (II, VII), *P. gingivalis* W50 (III, VIII), *P. gingivalis* E8 (IV, IX), or *T.d.* (V, X) and subsequent calcium response to SLIGRL-NH₂ (2 μ mol/L) or trypsin (2 nmol/L) 5 minutes later. XI represents the desensitization of PAR₂ receptor by two sequential treatments with trypsin for 5 minutes. Representative data from three experiments.

TLCK). Taken together, these results implicate the arginine-specific cysteine proteases RgpA and RgpB in PAR₂ activation.

The ability to activate PAR₂ exerted by *P. gingivalis* was compared with the effects exerted by *T. denticola*, a periodontal pathogen that expresses a serine protease in its outer sheath and several endo-acting peptidases in its culture supernatants. The *T. denticola* culture supernatant did not cause calcium signal in PAR₂-transfected cells (Figure 1B, V). However, the trypsin response of PAR₂-transfected cells pre-exposed to *T. denticola* culture supernatant was diminished (Figure 1B, X), whereas the

Figure 2. Proteolytic activity in chamber fluid samples from WT mice inoculated with *P. gingivalis* 33277, *P. gingivalis* W50, or *P. gingivalis* E8 preincubated or not with 10 nmol/L SBTI, 1 mmol/L PMSF, 1 mmol/L TLCK, or 1 mmol/L leupeptin. Means $\pm$ SEM, $n = 8$ (group). *Significant difference versus proteolytic activity generated by control treatment. †Significant difference versus proteolytic activity generated by *P. gingivalis* 33277 treatment in the same conditions. ‡Significant difference versus proteolytic activity generated by *P. gingivalis* W50 treatment in the same conditions. §Significant difference versus proteolytic activity generated by the same sample without protease inhibitor treatment.

response to the peptide agonist SLIGRL-NH₂ was similar to that of cells only exposed to the peptide (Figure 1B, V). This suggests that proteases released in the supernatant of *T. denticola* cultures do not activate PAR₂ but may disarm the receptor for subsequent activation by trypsin.

P. gingivalis Infection Resulted in Release of Proteolytic Activity

We hypothesized that PAR₂ can be activated upon *P. gingivalis* infection, thereby participating in the host response to infection. To this end, we first evaluated whether *P. gingivalis* infection provoked the release of proteolytic activity. Trypsin-like activity was tested in fluids collected from skin chambers after inoculation of *P. gingivalis* 33277, *P. gingivalis* W50, *P. gingivalis* E8, or saline. In Figure 2, WT mice showed a significant ($P < 0.001$) increase with regards to the proteolytic activity of chamber fluids after inoculation with *P. gingivalis* 33277, *P. gingivalis* W50, or *P. gingivalis* E8 when compared to chamber fluid from mice injected with saline (control). Yet, the proteolytic activity from samples of *P. gingivalis* E8-treated mice was significantly decreased when compared to the proteolytic activity found in chamber fluids after inoculation with *P. gingivalis* 33277 or *P. gingivalis* W50. Preincubation with the serine protease inhibitors SBTI or PMSF led to a significant reduction of the proteolytic activity on *P. gingivalis* 33277, *P. gingivalis* W50, or *P. gingivalis* E8 infection, whereas preincubation with TLCK or leupeptin showed a significant decrease only in the proteolytic activity of samples from *P. gingivalis* 33277-infected mice. In addition, systemic treatment of mice with the serine protease inhibitor SBTI significantly ($P < 0.05$) decreased the proteolytic activity because of *P. gingivalis* 33277 inoculation (mean $\pm$ SEM, 0.44 ± 0.23 versus 1.56 ± 0.21 for *P. gingivalis* 33277 + SBTI and *P. gingivalis* 33277 alone, respectively, data not shown in the figure).

PAR₂ Is Activated by *P. gingivalis* Infection-Induced Proteolytic Activity

To determine whether or not proteolytic activity released on *P. gingivalis* infection could cleave PAR₂, we examined the effects of *P. gingivalis*-infected chamber fluid samples on subsequent intracellular calcium [Ca²⁺]_i mobilization by SLIGRL-NH₂ in cultured cells transfected with PAR₂ (PAR₂-KNRK). We observed that chamber fluid samples (40 μ l) collected from WT mice after subcutaneous challenge with *P. gingivalis* 33277 or *P. gingivalis* W50 provoked calcium mobilization in PAR₂-KNRK but not in nontransfected (KNRK) cells (data not shown). Fluids collected from *P. gingivalis* 33277- or *P. gingivalis* W50-infected mice but not from *P. gingivalis* E8-injected mice were able to significantly ($P < 0.05$) reduce calcium mobilization in PAR₂-transfected KNRK cells generated by SLIGRL-NH₂ (mean $\pm$ SEM, 12.38 ± 1.35 , 12.35 ± 1.55 , 22.10 ± 2.12 for *P. gingivalis* 33277, *P. gingivalis* W50, and *P. gingivalis* E8 treatment, respectively; Figure 3), as compared to the effects of saline-injected chambers (mean $\pm$ SEM, 29.13 ± 0.30). Preincubation of the *P. gingivalis* 33277-infected or *P. gingivalis* W50-infected chamber samples with SBTI or PMSF significantly increased the subsequent calcium response to SLIGRL-NH₂ (Figure 3), thereby inhibiting the desensitization to SLIGRL-NH₂ response. Preincubation of chamber fluid samples with TLCK or leupeptin also provoked a significant increase in the subsequent calcium response to SLIGRL-NH₂, but only in samples from *P. gingivalis* 33277-infected mice.

Fluid samples collected from mice treated with both *P. gingivalis* 33277 and SBTI did not provoke a significant reduction in the subsequent calcium signal response to SLIGRL-NH₂ (mean $\pm$ SEM, 21.56 ± 1.72 ; data not shown), similar to the samples from *P. gingivalis* E8-treated and control-treated animals. Collectively, these results demonstrated the presence of PAR₂-activating proteases upon infection by *P. gingivalis*.

Figure 3. Effect of preincubation of chamber fluids collected from *P. gingivalis* 33277-, *P. gingivalis* W50-, or *P. gingivalis* E8-infected mice with 10 nmol/L SBTI, 1 mmol/L PMSF, 1 mmol/L TLCK, or 1 mmol/L leupeptin on the subsequent calcium response generated by the PAR₂ agonist SLIGRL-NH₂ (2 μ mol/L). Means $\pm$ SEM from eight separate experiments. *Significant difference ($P < 0.05$) versus calcium response generated by the same sample without protease inhibitor treatment. Increases in intracellular calcium were monitored as a percentage (% A23187) of the fluorescence emission (E530) caused by 2- μ mol/L concentrations of the calcium ionophore A23187.

Figure 4. Inflammatory effects of subcutaneous chamber infection with *P. gingivalis* 33277 in WT and PAR₂^{-/-} mice. Means $\pm$ SEM, $n = 10$ (group). **A:** Inflammatory cell counts; **B:** PGE₂ levels; **C:** IFN- γ levels; **D:** IL-1 β levels; **E:** IL-6 levels; and **F:** IL-10 levels.

PAR₂ Activation Plays a Pivotal Role in the Host Response to *P. gingivalis* Infection

Next, we evaluated whether PAR₂ activation affected the host response to *P. gingivalis* infection, by comparing the inflammatory effects of subcutaneous chamber infection with *P. gingivalis* 33277 in WT and PAR₂^{-/-} mice. Figure

4A shows that subcutaneous challenge with *P. gingivalis* led to a significant ($P < 0.001$) increase in inflammatory cell infiltration when compared to saline treatment (day 1 after saline or *P. gingivalis* injection) in WT mice. *P. gingivalis*-induced increased inflammatory cell counts were significantly diminished in PAR₂^{-/-} mice compared to WT. The inflammatory cell count was not significantly

Figure 5. PAR₂ activation role in *P. gingivalis*-induced periodontitis. **A:** Alveolar bone loss at 42 days after infection. Means $\pm$ SEM, $n = 10$ (group). Mandibular lingual aspect of alveolar bone loss in *P. gingivalis*-infected PAR₂^{-/-} (**B**) and WT (**C**) mice at 42 days after infection. **D:** Alveolar bone loss at 60 days after infection. Means $\pm$ SEM, $n = 10$ (group). Mandibular lingual aspect of alveolar bone loss in *P. gingivalis* infected PAR₂^{-/-} (**E**) and WT (**F**) mice at 60 days after infection. The **black arrows** indicate the areas of greater loss of alveolar bone.

increased in *P. gingivalis*-injected PAR₂^{-/-} mice compared with saline-injected PAR₂^{-/-} mice (Figure 4A).

Subcutaneous challenge of WT mice with *P. gingivalis* led to significant ($P < 0.05$) increases in PGE₂, IFN- γ ,

IL-1 β , and IL-6 levels when compared with saline treatment (Figure 4, B-E). No significant difference was observed between WT mice and PAR₂^{-/-} mice after saline or *P. gingivalis* injection for PGE₂ (Figure 4B) or IL-6 levels

(Figure 4E). However, although a significant increase in PGE_2 was observed on *P. gingivalis* infection in WT mice, this increase was not present in $\text{PAR}_2^{-/-}$ mice. Further, $\text{IFN-}\gamma$ and $\text{IL-1}\beta$ levels were significantly reduced in $\text{PAR}_2^{-/-}$ mice treated with *P. gingivalis* compared with WT mice. For $\text{IFN-}\gamma$, the cytokine level in $\text{PAR}_2^{-/-}$ mice was similar to noninfected mice (saline injected) (Figure 4C). No significant difference was observed between saline- or *P. gingivalis*-treated WT or $\text{PAR}_2^{-/-}$ mice for the levels of IL-10 release (Figure 4F). Evidently, PAR_2 activation contributes to the host response to *P. gingivalis* infection in the anaerobic chamber model in mice, causing the release of IL-1 and $\text{IFN-}\gamma$ and stimulating to the recruitment of inflammatory cells.

PAR₂ Activation Plays a Pivotal Role in P. gingivalis-Induced Periodontitis

Finally, we evaluated whether PAR_2 activation impacts *P. gingivalis*-induced periodontitis in mice. Satisfactory outcome of the experimental periodontitis model was confirmed by an increased bone loss after 42 (Figure 5, A–C) and 60 days (Figure 5, D–F) after *P. gingivalis* administration. Both in WT and $\text{PAR}_2^{-/-}$ mice, *P. gingivalis* infection caused significant alveolar bone loss. However, $\text{PAR}_2^{-/-}$ mice showed significantly less alveolar bone loss at both time points compared with WT mice after oral infection.

Discussion

Trypsin-like proteolytic activity is able to cleave the N-terminal peptide of PAR_2 , providing a newly exposed N-terminal end that functions as a tethered ligand, activating the receptor and resulting in intracellular signaling by G_q proteins.³ Pancreatic and extrapancreatic (endothelial and epithelial) trypsins, coagulation factors (FVIIa-FXa), mast cell tryptase, leukocyte proteases (neutrophil proteinase 3), and membrane-tethered serine protease-1 are some of the endogenous proteases that cleave and activate PAR_2 .^{9,12,13} In addition to endogenous host enzymes, the arginine-specific proteinases, gingipains-R (HRgpA and RgpB) derived from *P. gingivalis*, have also been identified as potential activators of PAR_2 . *In vitro* studies have reported that gingipains-R can activate PAR_2 in human neutrophils,²⁶ human oral epithelial cells,⁹ and in primary rat calvarial osteoblast-like cells¹⁸ through the induction of a dose-dependent increase in $[\text{Ca}^{2+}]_i$. In fact, gingipains are considered major virulence determinants of the bacterium,^{27–30} including their activity in periodontal breakdown, disruption of host defense mechanisms, and loss of viability of human fibroblasts and endothelial cells.

In the present study, we used KNRK cells transfected or not with PAR_2 combined with desensitization experiments using previous exposure to PAR_2 agonist to evaluate the role of bacterial proteases from *P. gingivalis* on PAR_2 activation. We show that *P. gingivalis* culture supernatants provoke Ca^{2+} mobilization in PAR_2 -KNRK cells and decrease the subsequent responses to the two PAR_2

agonists SLIGRL-NH₂ or trypsin. In addition, we demonstrate that after desensitization of the receptor by trypsin, the addition of *P. gingivalis* culture supernatant did not cause calcium response in PAR_2 -KNRK cells, excluding the possible concurrent activation of other receptors. Because trypsin signal to PAR_2 -transfected KNRK cells is exclusively mediated by PAR_2 ,³¹ our results demonstrate unequivocally that *P. gingivalis* supernatants and trypsin act on the same receptor, namely PAR_2 .

P. gingivalis produces at least eight different endopeptidases and a number of exopeptidases that belong to the cysteine-, serine-, and metallo-classes of peptidases.²⁹ It is well documented that among these enzymes, the cysteine proteinases, arginine-gingipain (Rgp) and lysine-gingipain (Kgp), which specifically cleave after arginine and lysine, respectively, have trypsin-like activity and are responsible for most of *P. gingivalis*-associated proteolytic activity.³² The gingipains are associated with both the cell surface and the outer membrane vesicles of the bacterium and can be produced in large amounts.³³ In the present study, Arg-gingipain-specific involvement in PAR_2 activation was delineated by using the culture supernatants of *P. gingivalis* W50 (WT) and E8 (RgpA/RgpB double knockout) strains. We showed the lack of PAR_2 activation by the Arg-gingipain knockout strain and inhibition of PAR_2 activation by incubating WT *P. gingivalis* culture supernatants with serine/cysteine inhibitors. These results, which evidence the capacity of *P. gingivalis*-released RgpA and RgpB in activating PAR_2 , are in agreement with previous studies in the literature that show that the purified gingipains-R (HRgpA and RgpB) are able to activate PAR_2 .^{9,18,21,26}

Recognizing the polymicrobial etiology of periodontitis, we compared the ability exerted by *P. gingivalis* to activate PAR_2 with the activity of *T. denticola*, an important periodontal pathogen that co-habits and forms biofilms with *P. gingivalis* and that also expresses proteinases with trypsin-like activity.^{29,34} Unlike *P. gingivalis*, *T. denticola* culture supernatant did not activate PAR_2 , as it did not cause calcium signaling and did not desensitize PAR_2 activation by SLIGRL-NH₂. Notably, both *T. denticola* outer membranes and its major outer sheath protein are known to cause calcium mobilization in fibroblasts, because of influx through the plasma membrane, but also to suppress subsequent agonist-induced calcium release from internal stores.^{35,36} In this study, the *T. denticola* culture supernatant neither stimulated a calcium signal nor desensitized PAR_2 activation by SLIGRL-NH₂. Yet, *T. denticola* seemed to disarm the receptor because subsequent activation by trypsin was inhibited. This result suggests a possible inhibitory effect of *T. denticola* proteases or peptidases on proteolysis-mediated PAR_2 activation. This disarming effect could be due to the subtilisin family serine protease dentilisin (PrTP), which is known to be shed with outer sheath vesicles into the culture medium in aging cultures. Similar observations have been made for *Pseudomonas aeruginosa* elastase, which disarms PAR_2 for subsequent activation by trypsin but not by the synthetic receptor activating peptide SLIGKV-NH₂.³⁷ It can be hypothesized that some pathogens may silence PAR_2

functions and avoid the organization of an effective host inflammatory response.

In addition to finding that culture supernatants of *P. gingivalis* were able to signal to PAR₂, we also show that increased proteolytic activity was observed in subcutaneous chambers on infection by *P. gingivalis* (Figure 2). Our results suggest that not only can Arg-gingipains be responsible for the proteolytic activity observed on infection but serine proteases might also be involved. First, infection by the gingipain RgpA/RgpB-defective mutant E8 also provoked an increased proteolytic activity in infected chambers, although this activity was significantly lower than what was observed after WT strain infection. Second, serine protease inhibition by SBTI, a known reversible inhibitor of trypsin, chymotrypsin, and elastase, and by PMSF, an irreversible serine inhibitor that does not affect gingipains, resulted in a significant decrease in the proteolytic activity of *P. gingivalis*-infected subcutaneous chambers. Mixed serine/cysteine protease inhibitors (TLCK and leupeptin) were not more effective at reducing *P. gingivalis* infection-induced proteolytic activity than serine protease-only inhibitors such as PMSF. Similar results were obtained in terms of PAR₂ activation, in which serine protease inhibitors (SBTI and PMSF) were as effective or more effective as serine/cysteine protease inhibitors (leupeptin or TLCK) to inhibit PAR₂ desensitization by *P. gingivalis*-infected chamber fluids. At this stage, it is not clear whether the serine proteases present in chamber fluids of *P. gingivalis*-infected mice were generated by the host or by the bacteria itself. *P. gingivalis* infection could trigger the host to generate PAR₂-activating proteases as it was recently demonstrated for *Citrobacter rodentium* infection in mouse colon.³⁸ It is also possible that the proteolytic activity observed in chamber fluids from *P. gingivalis*-infected mice is due solely to the host inflammatory reaction and is independent of the cause that triggers inflammation. It is interesting to note that infection by the RgpA/RgpB double-mutant E8 caused significantly less proteolytic activity in chamber fluids than WT strains and that E8-derived chamber fluids were not able to desensitize PAR₂ activation. This suggests that the presence of PAR₂-activating proteases in infected tissues might also depend on the virulence of the infection (E8 being less virulent than the other two strains, W50 and 33277) and potentially the release of Arg-gingipains. It is possible that Arg-gingipains in the subcutaneous chambers activate proteases of the coagulation cascade, which then could account for the increased proteolytic activity in the chambers of W50 and 33277 compared to Arg-gingipain mutant E8. As a matter of fact, studies have shown that *P. gingivalis* Arg-gingipains are able to activate prothrombin and coagulation factor X.^{39,40} Interestingly, PAR₂ has been described as the endogenous receptor mediating the effects of factor Xa in endothelial cells.⁴¹ Therefore, *P. gingivalis* Arg-gingipains could account for PAR₂ activation both directly (by cleaving the receptor) and indirectly (by inducing the release of PAR₂-activating proteases).

A number of studies have suggested a dual role for PAR₂ in inflammatory processes. Although PAR₂ activation seems to be involved in leukocyte migration, inflam-

mation of joints, skin, and kidney, and allergic inflammation of airways,³ it has also been linked to some protective anti-inflammatory activities via the epithelium and vascular endothelium in the airways,¹⁴ in the mucosal tissues of the gastrointestinal tract,¹⁵ or in response to cardiovascular injury.⁴² Conversely, the results from the present study clearly demonstrate that upon infection, PAR₂ plays a proinflammatory role in gingival tissues. Our data demonstrate that in the presence of *P. gingivalis*, PAR₂ activation leads to a significant increase in inflammatory cell infiltration, which was inhibited by SBTI and significantly reduced in PAR₂-deficient (PAR₂^{-/-}) mice. In addition, increased levels of prostaglandin E₂, IFN- γ , IL-6, and IL-1 β , important mediators of alveolar bone loss, were found in chambers of *P. gingivalis*-treated mice compared with control saline-treated mice. All these inflammatory mediators, with the exception of IL-6, were either not increased or significantly inhibited in PAR₂^{-/-} mice infected by *P. gingivalis*. Interestingly, although IL-6 production was significantly increased in subcutaneous chambers on *P. gingivalis* infection, no difference was found with regards to IL-6 levels released on *P. gingivalis* infection in PAR₂^{-/-} mice compared to WT mice. This result suggests that IL-6 production is probably not mediated by PAR₂ activation. Conversely, the study by Loubarkos and colleagues⁹ has shown in an oral epithelial cell line that expresses both PAR₁ and PAR₂ that treatment with selective agonists of PAR₁ and PAR₂, or Arg-gingipain, induced secretion of IL-6. It could be hypothesized that *in vivo* gingipain might act preferentially on PAR₁ rather than on PAR₂ to induce IL-6 production. Additionally, *P. gingivalis* may induce IL-6 production in the host by a mechanism that does not involve proteolytic activity *in vivo*.

The present study also provides evidence for an important role of PAR₂ in alveolar bone loss in mice orally challenged with *P. gingivalis* because a significant prevention of alveolar bone loss was verified in PAR₂^{-/-} mice at 42 and 60 days after infection. These results clearly show that PAR₂ is activated upon *P. gingivalis* infection, in which it contributes to the host inflammatory response. PAR₂-mediated activation of cells during infection is an intriguing new mechanism in bacterial pathogenicity that remains to be studied in depth. Because PAR₂ is a one-shot receptor being desensitized after proteolysis, it can be considered as an emergency or sensor mechanism of the environment, which is activated on infection. However, its possible role in the maintenance of inflammation cannot be overlooked because PAR₂ activation mediates the release of inflammatory mediators that, in theory, can up-regulate PAR₂ expression,⁴³ therefore resulting in an ultimate increased severity of the disease.

Interestingly, a recent paper by Tancharoen and colleagues,⁴⁴ reported that the cysteine proteinase RgpB from *P. gingivalis* elicits the release of neuropeptides by human dental pulp cells through PAR₂ activation, therefore suggesting its role also in pulp inflammation. These data also suggest that PAR₂ activation in response to *P. gingivalis* infection could organize an analogous neurogenic inflammatory response.

High levels of proteolytic activity have been found in gingival crevicular fluid from periodontal pockets, where a mixture of endogenous host enzymes and bacterial proteases combine to mediate degradation of connective tissue. Among these enzymes, neutrophil serine proteinase 3, mast cell tryptase, and gingipain have been isolated from the periodontal environment and are known to activate PAR₂.²⁸ Our present study further suggests a role for mixed endogenous and bacterial proteases in the pathophysiology of periodontitis. It identifies the activation of PAR₂ as one of the significant target of periodontitis-associated proteases and as a major factor in pathogenesis. Therapies focusing on the inhibition of proteinases or, more specifically, the use of PAR₂ antagonists, may constitute an important approach for the modulation of an infectious pathology such as periodontal inflammatory disease.

Acknowledgments

We thank Dr. Joe Aduse-Opoku for providing the strains W50 and E8 of *P. gingivalis*; Drs. Steeve Houle, Kristina K. Hansen, and Morley Hollenberg for expert assistance with cell culture and calcium signaling analysis; Dr. Thomas J. Louie's laboratory personnel for expert assistance with *P. gingivalis* culture; Dr. John L. Wallace and Webb McKnight for their valuable assistance in preparing the macroscopic figures; and Kevin Chapman and Laurie Cellars for technical assistance in general.

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