Plasmin Is Essential in Preventing Periodontitis in Mice

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Periodontitis involves bacterial infection, inflammation of the periodontium, degradation of gum tissue, and alveolar bone resorption, which eventually leads to loss of teeth. To study the role of the broad-spectrum protease plasmin in periodontitis, we examined the oral health of plasminogen (Plg)-deficient mice. In wild-type mice, the periodontium was unaffected at all time points studied; in Plg-deficient mice, periodontitis progressed rapidly, within 20 weeks. Morphological study results of Plg-deficient mice revealed detachment of gingival tissue, resorption of the cementum layer, formation of necrotic tissue, and severe alveolar bone degradation. IHC staining showed massive infiltration of neutrophils in the periodontal tissues. Interestingly, doubly deficient mice, lacking both tissue- and urokinase-type plasminogen activators, developed periodontal disease similar to that in Plg-deficient mice; however, mice lacking only tissue- or urokinase-type plasminogen activator remained healthy. Supplementation by injection of Plg-deficient mice with human plasminogen for 10 days led to necrotic tissue absorption, inflammation subsidence, and full regeneration of gum tissues. Notably, there was also partial regrowth of degraded alveolar bone. Taken together, our results show that plasminogen is essential for the maintenance of a healthy periodontium and plays an important role in combating the spontaneous development of chronic periodontitis. Moreover, reversal to healthy status after supplementation of Plg-deficient mice with plasminogen suggests the possibility of using plasminogen for therapy of periodontal diseases. (*Am J Pathol* 2011, 179:819–828; DOI: 10.1016/j.ajpath.2011.05.003)

Periodontal diseases comprise all of the diseases that affect one or more periodontal tissues. Most periodontal diseases are inflammatory diseases, and, depending on severity, they can be broadly divided into gingivitis and periodontitis.¹ Gingivitis is mild reversible inflammation of

the gums caused by bacterial plaque that accumulates on teeth at the gingival margin. Gingivitis affects >80% of the adult population in the United States and is regarded as one of the most common human diseases.² In some patients, gingivitis develops into periodontitis (ie, a severe and chronic inflammatory disease affecting tissues that support and anchor the teeth, such as alveolar bone, periodontal ligament, and cementum of the root). The destruction of the tooth-supporting periodontium eventually leads to loss of teeth. Periodontitis affects approximately 30% to 40% of adults in Europe and the United States,^{3,4} and the tissue destruction caused by periodontitis is generally regarded to be irreversible.

Periodontal diseases are caused by an imbalance in interplay between biofilm-forming bacteria on the tooth surface and the host immune response.¹ Invading bacteria may induce tissue damage by releasing compounds, such as lipopolysaccharides, that have a profound effect on many cell types, including fibroblasts, osteoblasts, and osteoclasts.^{5,6} In addition, lipopolysaccharides activate macrophages to release proinflammatory factors, such as tumor necrosis factor- α and ILs (IL-1, IL-6, and IL-8).^{7,8} However, the major cause of the bacterially induced destruction of periodontal tissue is activation of host inflammatory cells. One of the first events to occur during bacterially induced periodontal inflammation is accumulation of neutrophils in soft periodontal tissues. Neutrophils eliminate bacteria by phagocytosis and degranulation. However, some periodontopathogenic bacteria can evade the phagocytosis, resulting in continuous accumulation and degranulation of new neutrophils. Hydrolytic enzymes released from these neutrophils then cause irreversible damage to the gums, alveolar bone, cementum, and dentin, eventually leading to tooth loss.^{9,10}

Plasminogen (Plg) is an 88-kDa proenzyme that is synthesized mainly in the liver and secreted into the blood, where it circulates at a concentration of 2 μ mol/L. A considerable amount of Plg has also been found in the

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extracellular matrix (ECM).¹¹ Plg is the key component of the plasminogen activator (PA) system. It can be activated through a single proteolytic cleavage to become the active broad-spectrum serine protease plasmin. The activation is performed by either of two physiological PAs: tissue-type PA (tPA) or urokinase-type PA (uPA). Because of its high affinity for fibrin, plasmin was first regarded as a key enzyme in removing fibrin clots and preventing pathological blood clot formation.¹² However, it is known that plasmin also plays an important role in ECM remodeling through its ability to directly degrade ECM components, such as laminin, fibronectin, and proteoglycans,^{13–15} and indirectly activate matrix metalloproteinases.^{16–18} Many cells have various types of cell surface receptors for Plg, and plasmin activity generated on the cell surface plays a role for the migration of certain cells.^{19,20} Thus, plasmin is important for processes that require tissue remodeling, such as angiogenesis, metastasis, wound healing, and liver regeneration.^{13,14,20–22} In infectious diseases, Plg may play a dual role. On one hand, Plg is used by some bacteria to invade the host^{19,23,24}; on the other hand, plasmin may be important for host defense against infection.^{19,25,26} The processes previously mentioned, in which Plg plays an important role, resemble the processes involved in periodontitis. Therefore, it would be interesting to investigate the role of Plg in periodontitis.

Clinical Plg mutations have been reported in patients with ligneous gingivitis/periodontitis, which is a rare genetic periodontal disease with gingival enlargement and periodontal breakdown.^{27–30} In humans, inherited Plg mutations are divided into two types: type 1 (hypoplasminogenemia), in which the level of immunoreactive Plg is reduced in parallel with its functional activity; and type 2 (dysplasminogenemia), in which the level of immunoreactive Plg is normal (or slightly reduced) and the specific functional Plg activity is markedly reduced.³¹ Interestingly, patients with ligneous gingivitis/periodontitis are reported to have type 1 Plg deficiency. Recently, Klammt et al³² reported 23 new cases of severe hypoplasminogenemia, three of which were caused by novel mutations in the *Plg* gene. Although many individual cases have been reported, the lack of experimental models has made it difficult to dissect the exact molecular mechanisms behind pathogenesis of ligneous periodontitis.

Herein, we showed that Plg-deficient mice spontaneously develop severe periodontitis that resembles the human disease. More important, supplementation of Plg-deficient mice with Plg for 10 days resulted in complete elimination of inflammation, regeneration of soft periodontal tissues, and partial regrowth of alveolar bone. This makes Plg an attractive potential tool for treatment of periodontal diseases in humans.

Materials and Methods

Animals

Plg-deficient mice³³ were backcrossed for 10 generations with mice that had a C57BL/6 genetic background. To generate mice for experiments, Plg-heterozygous

mice were intercrossed; and the wild-type, heterozygous, and Plg-deficient progeny were studied. The genotype of these mice was determined by measuring plasma levels of plasminogen, as previously described.³⁴ Mice deficient in tPA and uPA were backcrossed for 10 generations with C57BL/6 mice.³⁵ Then, uPA and tPA heterozygous mice were intercrossed to generate the tPA/uPA doubly deficient mice. The genotype of these mice was determined by PCR analysis, as previously described.³⁶ The mice were kept on a 12-hour light/12-hour dark cycle and were fed standard chow and water ad libitum. All animal experiments were approved by the Regional Ethical Review Board of Umeå University, Umeå, Sweden.

Tissue Preparation and Morphological Staining

At the end of individual experiments, mice of both sexes were sacrificed by decapitation. Lower jaws were dissected free from surrounding soft tissues and transferred to a phosphate-buffered 4% paraformaldehyde solution for 24 hours at 4°C. Afterward, the jaws were decalcified in 10% EDTA for 3 weeks. Decalcified jaw samples were embedded in paraffin, sectioned to obtain 5- μ m-thick slices, mounted on slides, stained with fast green–safranin O (ie, cartilage, cementum, and epithelium, orange-red; and bone and dentin, blue), and counterstained with hematoxylin (Histolab Products AB, Gothenburg, Sweden).

IHC Staining of Neutrophils and Fibrin

Tissue sections (5- μ m thick) were deparaffinized in xylene and hydrated through a series of graded ethanol-water dilutions. The sections were then washed with PBS, blocked with goat serum (Rat ABC Staining System; Santa Cruz Biotechnology, Santa Cruz, CA), and incubated with rat monoclonal antibodies to mouse lymphocyte antigen G6 (Ly-6G, 1:250; eBioscience, San Diego, CA) or goat antiserum to mouse fibrinogen (Nordic Immunology, Tilburg, the Netherlands). Primary antibodies were detected with biotinylated anti-rat antibodies (Rat ABC Staining System, Santa Cruz, CA) and anti-goat antibodies (ABC Staining System; Santa Cruz Biotechnology). Diaminobenzidine substrate (Vector Laboratories, Peterborough, UK) was used for visualization. To control nonspecific staining, tissue sections were treated as previously noted, but the anti-Ly-6G primary antibody was omitted. Finally, the sections were counterstained with hematoxylin and the preparations were mounted with xylene-based Pertex mounting medium (Histolab Products AB). Images were obtained using a Leica DML microscope with a Leica D300F camera (Leica Microsystems GmbH, Wetzlar, Germany).

Quantification of Necrotic Tissue Area and Level of Alveolar Bone

Quantitative measurements of necrotic tissue and alveolar bone level were performed blindly on 100-fold magnified pictures of sections stained with safranin O, using

Table 1. Number of Mice Analyzed Morphologically in Each Age Group

Age group (weeks)	No. of Plg-deficient mice	No. of wild-type mice
4–8	28	15
9–12	13	9
13–16	16	13
17–20	13	12

AxioVision software version 4.2 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Alveolar bone degradation and the associated area of necrotic tissue were monitored between molars 1 and 2. The area on tissue sections where cells were morphologically indistinguishable was regarded as necrotic tissue and measured (in μm^2). The alveolar bone level was defined by measuring the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest. Data were analyzed by two-tailed Student's *t*-test, and differences were considered significant at $P < 0.05$.

Reconstitution of Plasminogen in Plg-Deficient Mice

Plg-deficient mice were randomly assigned to four experimental groups. Two groups, consisting of five mice aged 9 to 12 weeks and four mice aged 17 to 20 weeks, were injected i.v. daily with 1 mg of human plasminogen (Glu-plasminogen; Omnio AB, Umeå, Sweden) in 100 μL of PBS. In parallel, two control groups, five mice aged 9 to 12 weeks and four mice aged 17 to 20 weeks, were injected with 100 μL of PBS. All of the injections were performed i.v. on a daily basis for 10 days.

Alkaline Phosphatase Staining

Alkaline phosphatase staining was performed on paraffin-embedded tissue sections following a previously described method.³⁷ Briefly, dissected jaws were fixed for 24 hours in 2% paraformaldehyde containing 0.075 mol/L lysine and 0.01 mol/L sodium periodate solution (pH 7.4). After fixation, the specimens were washed for 12 hours with increasing concentrations of glycerol in PBS (step-wise up to 15%). The tissues were then decalcified in 0.4 mol/L EDTA solution (pH 7.3) containing 0.3 mol/L NaOH and 15% glycerol for 2 weeks and further washed to remove EDTA and glycerol. Finally, the jaw samples were embedded in paraffin, sectioned to 5- μm -thick slices, mounted on slides, deparaffinized, hydrated through a graded ethanol series, and preincubated in 1% magnesium chloride overnight. The sections were then incubated with alkaline phosphatase substrate solution (Fast Red TR/Naphthol AS-MX; Sigma-Aldrich, St. Louis, MO) for 1 hour and counterstained with methyl green (Vector Laboratories, Burlingame, CA).

Results

Plg-Deficient Mice Spontaneously Develop Severe Periodontitis

To investigate the condition of oral health in wild-type and Plg-deficient mice of different ages, mice were divided into four age groups (ie, 4 to 8, 9 to 12, 13 to 16, and 17 to 20 weeks) and morphological analysis of lower jaw sections was performed. The number of mice analyzed in each age group is shown in Table 1. No pathological changes were observed in periodontal tissues of wild-type mice up to the age of 20 weeks. As shown in Figure 1, A, C, E, and G, there was no inflammation in gum tissue, no alveolar bone degradation, and no detachment of tissue from teeth. Plg-deficient mice showed generally healthy periodontal tissues at the age of 6 weeks (Figure 1B). However, in older mice, the formation of necrotic tissue in the gum and the degradation of bone septa were observed. This was already apparent at the age of 11 weeks (Figure 1D) and had progressed by the age of 15 weeks (Figure 1F). At the age of 20 weeks (Figure 1H), all Plg-deficient mice showed severe periodontitis: the gum tissue was detached from the teeth and had become

Figure 1. Plg-deficient, but not wild-type, mice spontaneously develop periodontal disease. Paraffin sections of 6-, 11-, 15-, and 20-week-old wild-type mice (A, C, E, and G, respectively) and Plg-deficient mice (B, D, F, and H, respectively) were stained with green-safranin O and counterstained with hematoxylin. N indicates necrotic tissue; M1, M2, and M3, molars 1, 2, and 3, respectively; and black horizontal line, alveolar bone crest. Original magnification: $\times 50$ (A–H); $\times 200$ (insets).

Figure 2. Plg deficiency leads to degradation of alveolar bone. Images of defleshed lower jaws of 18-week-old wild-type (A) and Plg-deficient (B) mice. The **solid line** marks the edge of alveolar bone in both genotypes. The **dashed line** in B indicates the presumed edge of alveolar bone under healthy conditions. **Arrows** indicate the resorption level of alveolar bone in a Plg-deficient mouse. M1, M2, and M3 indicate molars 1, 2, and 3, respectively. **C:** Quantification of alveolar bone level in wild-type and Plg-deficient mice of different ages. Alveolar bone degradation was monitored between molars 1 and 2 and is expressed as the distance between the CEJ and the alveolar bone crest (B. crest).

increasingly necrotic, the cementum layer was completely destroyed, and gingival fibers were not distinguishable.

Clinically, periodontitis in humans is characterized by the degradation of alveolar bone, which causes loose teeth and, in severe cases, loss of teeth. Figure 2 shows a comparison of alveolar bone in the lower jaws of wild-type and Plg-deficient mice at the age of 18 weeks. Although the alveolar bone in wild-type mice was intact and covered the roots of teeth (Figure 2A), the alveolar bone in Plg-deficient mice was severely degraded, exposing approximately two thirds of molar roots (Figure 2B). To quantify the alveolar bone level in wild-type and Plg-deficient mice, we measured the distance between the CEJ and the alveolar bone crest on sectioned tissue samples. As shown in Figure 2C, the level of alveolar bone crest was similar in wild-type and Plg-deficient mice in the 4- to 8-week age group. However, at the age of 9 to 12 weeks, alveolar bone was significantly lower in Plg-deficient mice than in wild-type mice; and the difference further increased with age. Taken together, these results clearly demonstrate that, in contrast to wild type mice, Plg-deficient mice spontaneously develop periodontitis that becomes more severe with age.

Extensive Neutrophil Accumulation in the Periodontal Tissue of Plg-Deficient Mice during Spontaneous Development of Periodontitis

Human periodontitis is classified as an infectious disease, and it is accompanied by massive infiltration of neutrophils into soft periodontal tissues.⁹ To determine whether the development of spontaneous periodontitis in Plg-deficient mice resembles human disease in this respect, we analyzed neutrophils in the periodontium of mice at the ages of 4, 11, 15, and 19 weeks (Figure 3). As shown by immunohistochemical (IHC) staining, only a few neutrophils were detected in periodontal samples from wild-type mice at all time points (Figure 3, A, C, E, and G). Also, 4-week-old Plg-deficient mice did not show any difference in neutrophil staining compared with wild-type mice of the same age (Figure 3B). However, more

neutrophils were detected in 11-week-old Plg-deficient mice (Figure 3D); and massive accumulation of neutrophils was seen in these mice at the ages of 15 and 19 weeks (Figure 3, F and H, respectively). Most of the neutrophil-specific immunoreactivity was observed in the gum tissue between molars, which is the area where necrosis was detected. This increased infiltration and accumulation of neutrophils in the gum tissue of Plg-deficient mice suggests that the lack of expression of Plg

Figure 3. Neutrophil accumulation in the gums of wild-type and Plg-deficient mice. Tissue sections from the lower jaws of wild-type mice (A, C, E, and G) and Plg-deficient mice (B, D, F, and H) of different ages (as indicated) were immunostained with antibodies to mouse lymphocyte antigen G6. M1 and M2 indicate molars 1 and 2, respectively. Original magnification, $\times 400$.

Figure 4. Fibrin accumulation in the gums of wild-type and Plg-deficient mice. Tissue sections from the lower jaws of wild-type mice (A, C, and E) and Plg-deficient mice (B, D, and F) of different ages (as indicated) were immunostained with antibodies to mouse fibrinogen. M1 and M2 indicate molars 1 and 2, respectively. The **black horizontal line** marks the alveolar bone crest. Original magnification, $\times 200$.

results in persistent and unresolved inflammation of the periodontal tissue.

Fibrin Deposits in Damaged Gum Tissue of Plg-Deficient Mice

In previous studies of Plg-deficient mice, fibrin deposits have been documented in some organs. Therefore, we performed IHC staining of fibrin in gum tissue of wild-type and Plg-deficient mice. There were no fibrin deposits observed in periodontal tissues of wild-type mice up to the age of 20 weeks (Figure 4, A, C, and E). Fibrin deposits were also not detected in the gums of Plg-deficient mice at the age of 4 to 5 weeks (Figure 4B). However, along with a progression of periodontal disease in Plg-deficient mice, we could detect fibrin staining, but only in areas of diseased and necrotizing gum tissue (Figure 4, D and F).

tPA/uPA Doubly Deficient Mice Spontaneously Develop Severe Periodontitis

To investigate whether the development of periodontitis in Plg-deficient mice was because of the lack of plasmin activity, we studied singly deficient mice lacking either tPA or uPA and doubly deficient mice lacking both tPA and uPA. Singly deficient mice lacking either tPA (Figure 5A) or uPA (Figure 5B) had healthy periodontal tissues at the age of 18 weeks with no sign of alveolar bone degradation (Figure 5D). However, doubly deficient mice

Figure 5. The tPA/uPA doubly deficient mice develop periodontal disease, whereas tPA and uPA single-deficiency mice remain healthy. Representative jaw sections from 18-week-old tPA single-deficiency mice (A) and uPA single-deficiency mice (B) and the tPA/uPA doubly deficient mice (C) were prepared and stained as described in the legend to Figure 1. N indicates necrotic tissue; M1, M2, and M3, molars 1, 2, and 3, respectively; **black horizontal line**, alveolar bone crest. **D:** Quantification of alveolar bone levels (B, crest) in wild-type and PA-deficient mice. *** $P < 0.0001$.

Figure 6. Plasminogen supplementation of Plg-deficient mice results in healing of periodontitis and reversion of pathological changes in soft periodontal tissues to a healthy condition. Plg-deficient mice in two age groups (as indicated) were injected i.v. with PBS (**A** and **B**) or 1 mg of human plasminogen (**C** and **D**) for 10 days. Then, jaw sections were prepared as described in the legend to Figure 1. **E** and **F**: Analogous sections of jaws from untreated wild-type mice are shown for comparison. N indicates necrotic tissue; M1, M2, and M3, molars 1, 2, and 3, respectively; black horizontal line, alveolar bone crest.

lacking both tPA and uPA (Figure 5C) showed severe changes in the periodontium, including necrosis, detachment of gum tissue from the molars, and severe degradation of the alveolar bone. The severity of periodontal disease in the tPA/uPA doubly deficient mice was similar to that in Plg-deficient mice comparing levels of alveolar bone degradation (Figure 5D and 2C, respectively). Considering that tPA and uPA are the two major physiological PAs, these results clearly show that plasmin activity is the essential factor for maintenance of healthy periodontal tissues in mice.

Systemic Supplementation of Plg-Deficient Mice with Plasminogen Cures Their Periodontal Disease

Because Plg-deficient mice spontaneously develop periodontitis that resembles the human disease, we wanted to know whether supplementation with Plg might reverse the pathological changes in the periodontium. Plg-deficient mice were divided into two groups: 9- to 12-week-old mice with early symptoms of periodontitis and 17- to 20-week-old mice with severe periodontitis. The mice in each group received supplementation daily with human Plg by i.v. injection (1 mg/100 μ L) for 10 days or received an injection with PBS as a control. As shown in Figure 6, A and B, the periodontium of all mice injected with PBS

had pathological changes that were typical of Plg deficiency at this age (compared with Figure 1, D and H, respectively). However, the periodontal tissues of all mice treated with human Plg (Figure 6, C and D) had significantly recovered. After 10 days of treatment, the periodontal tissue was generally healthy and appeared almost as healthy as in wild-type mice (Figure 6, E and F). In both age groups of Plg-deficient mice that received supplementation with Plg, periodontal regeneration involved reappearance of fibroblasts and connective tissue and realignment of collagen structures. This resulted in the formation and reattachment of epithelium and connective tissue fibers to the surface of the root (Figure 6, C and D). In addition, necrotic tissue was significantly reduced in all mice that received supplementation with Plg (Figure 6, C and D) compared with PBS-treated animals (Figure 6, A and B). Quantification of necrotic tissue clearly showed that all of the necrotic tissue had been removed in the Plg-treated mice aged 9 to 12 weeks and that only approximately 6% of the necrotic tissue remained in the mice aged 17 to 20 weeks, relative to the status of untreated Plg-deficient mice. In contrast, the amount of necrotic tissue had increased in corresponding mice that had been given PBS treatment (Figure 7, A and B).

Figure 7. Plasminogen supplementation of Plg-deficient mice leads to clearing of necrotic tissue and regrowth of alveolar bone. **A** and **B**: Quantification of necrotic tissue area in two age groups of Plg-deficient mice (as indicated) before and after supplementation with human plasminogen. The necrotic tissue area was quantified as described in the *Materials and Methods* section. **C** and **D**: Quantification of the alveolar bone levels in two age groups of Plg-deficient mice before and after supplementation with human plasminogen. The dotted line indicates the level of alveolar bone in wild-type mice. **E**: Alkaline phosphatase activity staining in jaw sections of untreated wild-type mice and Plg-deficient mice supplemented with PBS or human plasminogen. Original magnification, $\times 100$.

Periodontitis in Plg-deficient mice was characterized by severe alveolar bone degradation. As shown in [Figure 7, C and D](#), the distance between the CEJ and the alveolar bone crest in Plg-deficient mice of both age groups before treatment was significantly greater than for wild-type mice. PBS treatment of Plg-deficient mice resulted in further loss of alveolar bone: by approximately 20% and 7% in mice in the 9- to 12-week and the 17- to 20-week age groups, respectively. However, the distance between the CEJ and the alveolar bone crest in Plg-treated mice decreased by approximately 17% in 9- to 12-week-old mice and 25% in 17- to 20-week-old mice ([Figure 7, C and D](#), respectively), indicating that there was significant regrowth of alveolar bone in the plasminogen-treated mice. To confirm this finding, we performed staining for alkaline phosphatase activity, an enzyme that is known to be expressed by osteoblasts at the site of bone formation. As expected, there was no alkaline phosphatase activity at the alveolar bone crest in the PBS-treated mice ([Figure 7E](#)). However, the mice that received supplementation with Plg showed clear staining for alkaline phosphatase at the surface of alveolar bone, which resembled that in wild-type mice ([Figure 7E](#)). This indicates that Plg supplementation of Plg-deficient mice leads to the activation of osteoblasts in alveolar bone.

Discussion

People who experience Plg deficiency have a tendency to develop ligneous periodontitis.^{27,28} However, to our knowledge, the possible role that Plg might play in the maintenance of periodontal health had not been studied. Herein, we showed that Plg-deficient mice spontaneously develop periodontitis that progresses with age. The development of periodontitis in these mice resembles that in humans and is characterized by the formation of necrotic tissue, the influx of neutrophils, and the detachment of gingival tissue from the teeth, followed by severe degradation of alveolar bone. This contrasts with the situation in wild-type mice, which have a healthy periodontium irrespective of age. Therefore, Plg-deficient mice can be used as a new rodent model to study periodontitis. More important, systemic supplementation of Plg-deficient mice with human Plg for 10 days resulted in almost complete regeneration of soft periodontal tissues and significant regrowth of alveolar bone. These results indicate that Plg has an essential role in protecting against spontaneous development of chronic periodontal disease. Our results also suggest that Plg treatment may be a new therapeutic strategy to treat infectious periodontitis.

In previous studies, doubly deficient mice lacking both tPA and uPA show much more severe phenotypes than mice with a tPA or uPA single deficiency in liver injury³⁸ and wound healing³⁹ models. Herein, we showed that Plg-deficient mice and tPA/uPA doubly deficient mutant mice spontaneously develop periodontitis with a severity similar to that seen in Plg-deficient mice, whereas tPA and uPA single-deficiency mice had a normal and healthy periodontium ([Figure 5](#)). Because tPA and uPA are the major physiological activators of Plg, our results

indicate that active plasmin is essential for the prevention of periodontitis and that lack of plasmin activity results in the development of the disease. A likely explanation is that in the single-deficient mice, the remaining Plg activator could activate plasmin to the level that is required for prevention of periodontal disease.

Periodontal inflammation in humans is initiated by bacterial colonization and expansion at the site of attachment, between the gum tissue and the tooth, which stimulates the host defense and migration of inflammatory cells into periodontal tissues.^{9,10} Plg-deficient mice are more susceptible to systemic infection and bacterially induced arthritis than wild-type mice, and supplementation with Plg improves the bacterial killing ability.²⁶ Therefore, we estimated the number of bacteria in extracts prepared from homogenized mandibles from wild-type and Plg-deficient mice by plating on blood agar plates (data not shown). Our results revealed that the number of colony-forming units in extracts from Plg-deficient mice was approximately 100-fold higher compared with wild-type mice. We also treated Plg-deficient mice with amoxicillin by supplementing drinking water from birth until the mice were examined.⁴⁰ However, periodontium degradation in the amoxicillin-treated mice was comparable to nontreated Plg-deficient mice, suggesting that the amoxicillin treatment did not rescue the Plg-deficient mice from the disease. Future more extensive studies on mice bred in a pathogen-free environment will elucidate whether periodontitis in this mouse model is because of periodopathogens or related to defects in the immune defense.

Previous studies⁴¹ of Plg-deficient mice have revealed fibrin deposits in some tissues. However, in this study, we could only detect fibrin deposits in already diseased gum tissue ([Figure 4](#)). Thus far, the molecular mechanism behind the initiation of periodontal disease in Plg-deficient mice has not been analyzed in detail. Therefore, it cannot be excluded that fibrin may play a role in the development of the disease. One possibility is that fibrin, in synergy with bacteria, triggers inflammation and thereby contributes to the development of periodontal disease. The fact that only 32% of patients who have type 1 Plg deficiency develop ligneous gingivitis/periodontitis suggests that external triggers, such as infections and trauma, may play a role in the development of disease.^{29,32} Numerous ligneous gingivitis/periodontitis case studies^{42–45} in humans have shown intense infiltration of inflammatory cells, fibrin deposits, irregularly downward epithelium proliferation, and alveolar bone degradation. Unfortunately, bacterial determinations were not performed in those patients.

Plg-deficient mice show persistent inflammation and neutrophil migration to the infected gum tissue. Despite the presence of neutrophils in these mice, there was no or little tissue debridement leading to accumulation of necrotic tissue and, finally, to development of periodontitis ([Figures 1 and 3](#)). However, the administration of Plg to these mice resulted in quenching of inflammation and removal of necrotic tissue from the periodontal area ([Figures 6 and 7](#)). These results support the idea that Plg has a role in activation of the host defense system against bacterial invasion and suggest that the phagocytic func-

tion of neutrophils may be impaired in the absence of Plg.⁴⁶ In line with the present findings, a previous *in vivo* study,²⁶ performed in wild-type and Plg-deficient mice using a *Staphylococcus aureus*-induced arthritis model, revealed protective roles of Plg. The importance of leukocyte activity for the prevention of periodontitis is also supported by studies^{47,48} showing that impaired leukocyte function in mice and humans that are deficient in L-selectin and in humans with leukocyte adhesion deficiency 2 correlate with an early onset of progressive periodontitis.

The regeneration of periodontal tissues involves complex interactions between different cell types, the ECM, and local mediators, such as growth factors and cytokines. There are many conventional treatments that are used for periodontitis, but few of them result in complete regeneration of periodontal soft tissue.⁴⁹ As shown herein, the administration of Plg to Plg-deficient mice led to full regeneration of soft periodontal tissues. This involved removal of necrotic tissue and formation of new periodontal ligament fibers connecting epithelial and connective tissues to the root of the tooth (Figure 6). Similar to what has been described herein, Plg-driven tissue remodeling and healing were observed when Plg-deficient mice with bacterially induced arthritis received supplementation with Plg.²⁶ In that case, Plg supplementation resulted in removal of necrotic tissue and bacteria, regeneration of cartilage, and, finally, restoration of healthy morphological features in the knee joint. It is likely that the ability of plasmin to degrade ECM components directly or through activation of pro-matrix metalloproteinases plays an important role in both processes.^{18,50}

There appears to be no single therapeutic treatment that can reverse all pathological changes in soft and bone periodontal tissues. One of the major problems with treatment of periodontitis is the regeneration of alveolar bone. Most often, the healing of the periodontium is accompanied by rapid growth of epithelial cells that cover the root surface. This prevents the repopulation of osteogenic fibroblasts and osteoblasts on wounded periodontal bone and, therefore, prevents bone regeneration.^{51–55} In fact, few treatments of periodontitis stimulate the regrowth of alveolar bone to any great extent; and periodontal bone regeneration is still regarded to be more or less an illusion.⁴⁹ The most promising methods, such as guided tissue regeneration, enamel matrix protein treatment, and use of growth factors, are expensive and technically demanding. Surprisingly, in this study, we found that simple 10-day systemic supplementation of Plg-deficient mice with plasminogen resulted in the appearance of active osteoblasts and regrowth of alveolar bone by up to 25% (Figure 7).

The formation/regeneration of bone involves three different phases: resorption, bone formation, and mineralization. During the resorption phase, bone matrix at the site of the wound is degraded to prepare attachment sites for bone-building cells. Bone matrix degradation also results in release and activation of growth factors and chemoattractants that drive the adhesion and proliferation of osteoblasts during the regeneration process.⁵⁶ Plasmin activity is important for the resorption phase, by

degrading noncollagenous components of bone matrix.⁵⁷ Several studies^{14,58,59} have also shown that the PA system has the ability to activate pro-matrix metalloproteinases and that plasmin, in concert with matrix metalloproteinases, directly degrades components of the ECM, including fibronectin, collagen, proteoglycans, and laminin. However, we cannot exclude the possibility that plasmin also participates in bone formation. Plasmin can activate transforming growth factor β and release insulin-like growth factor from bone matrix, which may then independently stimulate osteoblast proliferation and activity.^{60,61} *In vitro*, plasmin can cleave osteocalcin,⁶² which is a bone-specific protein important for recruitment of osteoclasts and osteoblasts.^{63,64} Osteoblasts express tPA, uPA, PA inhibitor 1, and a cellular receptor for uPA.⁶⁵ Interestingly, the bone matrix of tPA/uPA doubly deficient mice contains elevated amounts of osteocalcin, fibronectin, and proteoglycans; and shows increased bone formation.⁵⁷ However, these studies were performed in mice at the age of 2 to 7 days. It remains unknown whether bone formation may be impaired in adult animals. Based on our results, Plg may have positive effects on bone formation, especially in pathological conditions such as periodontitis.

To our knowledge, there has been no established treatment for humans with ligneous periodontitis; these patients do not respond to surgical treatments or to application of heparin combined with antibiotics.⁶⁶ Surgical removal of gingival masses, in combination with warfarin and antibiotic treatment, was successfully used in one of the ligneous gingivitis cases.⁶⁷ Our current data show that plasminogen supplementation in Plg-deficient mice can heal periodontitis, and previously reported data^{68,69} on effective treatment of ligneous conjunctivitis with topical Plg strongly indicate that Plg treatment in ligneous gingivitis/periodontitis in humans could be a potential therapeutic application.

Taken together, our results have shown that plasmin plays a critical role in maintaining healthy periodontal tissues and that the absence of plasmin activity leads to periodontitis. Plg-deficient mice spontaneously develop periodontitis with pathological features similar to that in humans. Therefore, this mouse model can be used as a new model for studies of periodontal disease. Supplementation of diseased Plg-deficient mice with plasminogen leads to clearing of necrotic tissue, full regeneration of soft periodontal tissues, and significant regrowth of alveolar bone, suggesting that plasminogen may be an effective future therapy for the treatment of periodontitis in humans.

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