Cellular Mechanisms that affect Periodontal Destruction induced by Bacteria Infection in Diabetic and Non Diabetic Rats

Sandra Pacios Pujadó

Dipòsit Legal: B 18914 - 2014

CELLULAR MECHANISMS THAT AFFECT PERIODONTAL DESTRUCTION INDUCED BY BACTERIA INFECTION IN DIABETIC AND NON DIABETIC RATS

Sandra Pacios Pujadó, DDS MS

Universitat Internacional de Catalunya - University of Pennsylvania
School of Dental Medicine
Department of Periodontics

DOCTORAL THESIS

May 2013

Dr. José Nart
Universitat Internacional de Catalunya
School of Dental Medicine
Department of Periodontics

Dr. Dana T. Graves
University of Pennsylvania
School of Dental Medicine
Department of Periodontics

A mi marido,
por su tiempo,
dedicación y apoyo
durante todos estos años.
Porque todo este trabajo
ha sido posible gracias a él.
Gracias
por haber cuidado de mi
con tanto esmero,
por tu comprensión y
tu cariño.

ACKNOWLEDGMENTS

ACKNOWLEDGEMENTS

Quisiera dedicara la finalización de esta tesis doctoral a todas aquellas personas que de una manera u otra me han apoyado durante estos años y la han hecho posible.

En primer lugar, quiero expresar mi agradecimiento a mis directores de tesis. Al Dr. Dana T Graves, por haberme ayudado y depositado su confianza en mi desde que empecé el proyecto en su laboratorio. Gracias por haberme enseñado todo lo que sé como investigadora, por haberme guiado a través de estos años con tu saber y pasión por la investigación. También al Dr. José Nart, por haber estado siempre disponible en todo momento y ofrecido su apoyo en este trabajo. Gracias por tu amistad y por tu orientación profesional mucho antes de que este proyecto empezara. Gracias a los dos por vuestra confianza depositada en mí.

Quiero mostrar un agradecimiento especial a toda mi familia, que me han ayudado con su apoyo a realizar este proyecto tan lejos de ellos. Muy especialmente a mis padres MºTeresa y Vicenç, por su cariño, ánimo y optimismo y por suplir todo en lo que o no he llegado durante estos años. A todos mis hermanos, Maite, Pilar, Carmen, Vicenç, Toni, Robert, Marta, Mercè y Francesc, cuñados, Xavi, Jordi, Kiko y Sandra y a Gemma, Luis, Ramón y Ana, sobrinos, Roger, Anna, Martita, Aleix, Mariona, Cesc, Queralt y Xavito, que han participado directa o indirectamente en la elaboración de esta tesis.

También a mi otra parte de la familia, Iñaki y Marta, a mis cuñados, Álvaro, Gonzalo, Guille y Martita; y a Adriana y Leti, por estar pendiente de todos mis proyectos y por su apoyo y cariño durante todos estos años.

Asimismo, a todo el equipo de mi laboratorio. A Hemal, Ioanah, Bhaskar, Dong, Johnah, Kang, Oelisoa, Wenmei, Wendy, Tina, Maher, Marcelo, Rohinton, Jason… por haber estado trabajando a mi lado y ayudado en lo que necesitara desde los inicios de mi investigación. Algunos de los cuales ya han emprendido otros caminos y con los que he compartido los mejores y no tan buenos momentos de estos años, debo agradecerles que a pesar de lo difícil del camino han conseguido que sólo las risas acompañen nuestro trabajo diario. A Scott por su espíritu de servicio y ayuda.

También quiero expresar mi agradecimiento a todos los de la UIC que han estado pendientes de mi con mis emails casi diarios. Especialmente a Mercè Vila y a Javi Sanz, por su tiempo y por su ayuda en asesorías y dudas presentadas en la elaboración de la tesis.

Al Dr. Luís Giner, por impulsar el desarrollo de mi formación profesional y apoyarme en todo momento.

A mis amigas, Anna M, Lili, Núria, Mercè, Imma, Mariona, Adriana, Ichi, Miriam, Clara, MCarmen, Alicia, Mireia, Maria, Oona… porque aunque hayan sido unos años a la distancia, hemos compartido momentos muy especiales. Gracias porque de una manera u otra me habéis acompañado en este camino.

Gracias a todos por todo vuestro apoyo que ha hecho posible la elaboración de esta tesis doctoral. Gracias con todo mi cariño.

INDEX

INDEX

INTRODUCTION

INTRODUCTION

Periodontal disease is one of the most prevalent infectious diseases among the population in the world. The prevalence of this disease is difficult to estimate because of two factors; periodontal disease is not defined consistently between studies, and the age group examined is not consistent. It has been reported that between 5% to 20% of the population suffers from severe generalized periodontitis and that most adults are affected by mild to moderate periodontitis (1). For those individuals who are more susceptible, periodontitis is evident in adolescence and in early adult age (2). There are risk factors that contribute to incidence of the disease including: smoking, genetic predisposition, possibly psychosocial stress, diabetes and rare systemic diseases.

The control of periodontal disease is important in maintaining the integrity and function of the oral cavity. This disease consists of destructive non-reversible damage that compromises the supporting structure of the tooth, leading to loss of attachment and bone, which ultimately produces loss of the involved teeth. Periodontal disease is initiated by bacteria, and as such many studies have been conducted to evaluate the composition of the subgingival biofilm and identify key periodontal pathogens utilizing both cultivation and molecular methods. More than 700 bacterial species can be found in the oral cavity, yet only a small percentage of these are thought to initiate periodontal disease. The process of periodontitis involves colonizing the tooth surface and penetration of the bacteria and their products into the connective tissue, resulting in inflammation that leads to periodontal destruction. This inflammation may also limit the repair process (3). It is well-known that the inflammatory response rather than the direct pathologic effects of bacteria are pivotal in stimulating periodontal disease.

Periodontal disease in humans and experimental animal models is linked to both the innate and adaptive immune response. Neutrophils and macrophages may contribute to tissue injury by secreting different immune mediators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and receptor activator of nuclear factor kappa-B ligand (RANKL). These mediators are abundantly expressed in individuals with periodontal disease having increased levels in the gingiva and crevicular fluid (3). Animal studies have also established relationships between these cytokines and the periodontal breakdown (4). Both genetic deletion and specific inhibition of these cytokines have been

found to reduce periodontal disease progression (5, 6). The adaptive immune response is mediated by the generation of antigen-specific T and B cells. The generation of factors by lymphocytes including RANKL and interferon-γ (IFN-γ) has been shown to contribute to periodontal bone loss (7, 8).

Strong relationships have been described among different forms of periodontal disease and certain bacterial species. Socransky et al (9) grouped them in different complexes according to the relationship presented in the etiology of periodontal disease. Bacteria, such as *Porphyromonas gingivalis (P.g)*, *Tannerella forsythia (T.f)*, and *Treponema denticola (T.d)*, are characterized as the "red complex," which is highly associated with periodontal tissue destruction. Other bacteria, such as *Prevotella intermedia (P.I)*, *Campylobacter rectus (C.R)*, *Fusobacterium* species, *Peptostreptococcus micros*, and various *spirochetes*, have been implicated in the development of periodontitis (10). Localized aggressive periodontitis in particular seems to be characterized by specific infection with *Aggregatibacter actinomycetemcomitans (A.a)*, whereas *P.g* and *T.f* are more likely to be associated with chronic periodontitis (11, 12).

Localized aggressive periodontitis (LAgP), previously known as localized juvenile periodontitis, primarily affects adolescents and exhibits severe and rapid destruction of the supporting apparatus around first molars and central incisors (13). This predilection may be the result of selective colonization of the teeth. Nowadays it is still being determined the genetic basis of both aggressive and chronic periodontitis. There is evidence that parents, offspring and siblings of patients affected with aggressive periodontitis have a 50% higher risk of presenting with the disease (14). LAgP shows minimal signs of inflammation and plaque build-up (15), but is associated with rapid destruction of the periodontal ligament and alveolar bone, which may lead to tooth loss early in life. LAgP is usually detected and diagnosed during puberty in systemically healthy individuals (15). As reported above, the primary suspected pathogen associated with this form of periodontal disease is *A. actinomycetemcomitans*. Many studies have described the strong relationship between *A. actinomycetemcomitans* and LAgP, and have shown the efficacy of periodontal treatment in reducing its severity (15, 16). The presence of *A. actinomycetemcomitans* in periodontal pockets has also been considered indicative of future disease progression (17, 18).

A. actinomycetemcomitans is a small gram-negative coccobacillus that is non-motile. It grows singular, in pairs, or in small clumps and is variously described as facultatively anaerobic, microaerophilic, and capnophilic (19, 20). It is found in the oral cavity of man and other mammals (21, 22). Within the human oral cavity *A. actinomycetemcomitans* resides in the supragingival plaque, subgingival plaque, saliva, cheek mucosa, buccal mucosa, gingivae, tongue (dorsal and lateral surfaces), hard palate, and tonsils (23). The organism can be detected in the oral cavity of both diseased and healthy subjects, suggesting that different hosts might have varying levels of susceptibility and pathogenicity in different strains of *A. actinomycetemcomitans* (24, 25).

A. actinomycetemcomitans has been associated with non-oral infectious diseases such as endocarditis, infectious arthritis, osteomyelitis, subcutaneous and brain abscesses (26-29). Presently there is interest in exploring the possibility that periodontal disease may be a risk factor for the development of other diseases such as diabetes or cardiovascular diseases.

Even though the exact understanding of the *A. actinomycetemcomitans* pathogenesis mechanism has not been established, its relation with LAgP has been significantly investigated because of its increased prevalence in individuals with the disease and its diverse potential virulence characteristics such as leukotoxin, cytolethal distending toxin, multiple immune evasion mechanisms and unusual mechanisms for binding to host matrices and invading host cells (30-32).

Rat periodontitis models, such as the inoculation of *A. actinomycetemcomitans* bacteria or the placement of a ligature, have often been used to study the effects of the bacteria and periodontal disease progression (24, 33, 34). The use of the animal models can be effective in establishing a cause-and-effect relationship by applying inhibitors or activators or through the use of genetically modified animals. However, not all animal models can effectively explain every feature of a disease. Periodontal disease can be broken down into four major steps: colonization of the bacteria, invasion of bacteria into susceptible host, inflammatory response triggered by host-bacterial relations, which leads to damage of periodontal tissues, and the repair process that alleviates the tissue damage occurred during the entire process (34, 35). Therefore, it should be understood that one animal model might not satisfy every aspect of the disease. Rather it should be emphasized

whether the model is appropriate for the hypothesis that is under consideration.

Two rat models have been used in this study, the *A. actinomycetemcomitans* model and the periodontal ligature model. *A. actinomycetemcomitans* model is characterized by the infection of the animals with a rough strain of *A. actinomycetemcomitans* known to adhere to different surfaces. The periodontal ligature model consists in the application of ligatures around the 1st and 2nd molars that causes the accumulation of plaque. Both of these methods result in periodontal disease destruction.

Under normal circumstance inflammation resolves through an active process regulated by cellular signals. However, resolution of the inflammation is impaired in periodontal disease and it is aggravated by systemic conditions such as diabetes.

The aim of this research was to evaluate in the rats models the histologic and cellular response to *A. actinomycetemcomitans* infection, and how diabetes-enhanced TNF-α production and diabetes-enhanced apoptosis contribute to the periodontal disease progression and bone coupling.

HYPOTHESIS

HYPOTHESIS

Hypothesis 1:

- **Null hypothesis (H0):** *A. actinomycetemcomitans* infection in Wistar rats does not increase local and systemic inflammatory response, loss of attachment and osteoclasts numbers causing periodontal bone loss.
- **Alternative hypothesis (H1):** *A. actinomycetemcomitans* infection in Wistar rats increases local and systemic inflammatory response, loss of attachment and osteoclasts numbers causing periodontal bone loss.

Hypothesis 2:

- **Null hypothesis (H0):** The inflammation in diabetic animals will not lead to greater levels of apoptosis thereby interfering with the capacity to repair bone after induced bone loss.
- **Alternative hypothesis (H1):** The inflammation in diabetic animals will lead to greater levels of apoptosis thereby interfering with the capacity to repair bone after induced bone loss.

Hypothesis 3:

- **Null hypothesis (H0):** Adding a specific TNF blocker (pegsunercept) or caspase 3-blocker will not overcome the impact of diabetes and improve bone repair following periodontal bone resorption.
- **Alternative hypothesis (H1):** Adding a specific TNF blocker (pegsunercept) or caspase 3-blocer will overcome the impact of diabetes and improve bone repair following periodontal bone resorption.

Hypothesis 4:

- **Null hypothesis (H0):** The inflammatory response will not decrease in diabetic animals after antibiotic treatment due to a difficulty in downregulating the inflammatory response to resist infection.

- **Alternative hypothesis (H1):** The inflammatory response will decrease in diabetic animals after antibiotic treatment due to a difficulty in downregulating the inflammatory responses to resist infection.

Hypothesis 5:

- **Null hypothesis (H0):** The improved bone formation will not be reflected by an increase in the numbers of osteoblastic cells.
- **Alternative hypothesis (H1):** The improved bone formation will be reflected by an increase in the numbers of osteoblastic cells.

OBJECTIVES

OBJECTIVES

Main objective:

The aim of the present project is to evaluate the onset and progression of periodontal disease and its modified progression by diabetes.

Secondary objectives:

1. Evaluate if *A. actinomycetemcomitans* is a good model to study the onset and progression of inflammation in periodontal disease and how is modified by antibiotic treatment.
2. Evaluate how diabetes modifies the onset and progression of inflammation in periodontal disease and how is modified by antibiotic treatment.
3. Look at the resolution of the inflammation and how this affects in bone coupling.

RESULTS

RESULTS

A. actinomycetemcomitans-induced periodontal disease promotes systemic and local responses in rat periodontium.

A total of 44 Wistar rats were included in the study (Appendix 1-3). The rats were divided in 2 groups. The first group was baseline. Baseline rats did not undergo antibiotic treatment and were not exposed to *A. actinomycetemcomitans*. The second group received antibiotic in their water to suppress their natural bacterial flora. After antibiotic treatment, they were inoculated with *A. actinomycetemcomitans*. Post inoculation, the animals were euthanized 4, 5 and 6 weeks later. Part of the population of rats that were exposed with *A. actinomycetemcomitans* were supplemented with antibiotics for 4 days and were euthanized 1 and 2 weeks after (at 5 and 6 weeks time point) (Appendix 4).

To determine if the rats were infected with *A. actinomycetemcomitans*, PCR from soft and hard tissues of the oral cavities was performed at the time of euthanasia. 78% of the animals showed infection. No *A. actinomycetemcomitans* was observed in rats that were not inoculated (baseline rats).

Antibody levels against *A. actinomycetemcomitans* were also examined. Four weeks after the rats were inoculated 80% presented with significant levels of antibody titers. The antibiotic treatment did not diminish the levels of the antibody titers**.**

Total anaerobe bacterial counts were not any different in all comparison made.

Histologically loss of attachment (LOA) was measured. Four weeks after the infection LOA significantly increased and it was reversed with the antibiotic treatment ($p < 0.05$).

Local inflammatory response was tested by analyzing the number of PMNs in the gingival epithelium and with TNF-α expression in the gingival and junctional epithelium (JE). *A. actinomycetemcomitans* stimulated the expression of PMNs and TNF-α significantly ($p < 0.05$). Though antibiotic treatment did not have any effect in the PMNs

and in TNF-α expression in the gingiva, it promoted a 25% decrease in TNF-α expression in the JE ($p < 0.05$).

Osteoclasts percentage was also significantly increased after the inoculation of *A.actinomycetemcomitans* ($p < 0.05$). The group with antibiotic treatment did not show a significant change in osteoclast presence.

To measure the rat's systemic response to *A. actinomycetemcomitans*, leukocyte presence in lymph nodes and blood were examined. The only findings of statistical significance seen were a 1.6-fold increase in CD8+T count after *A. actinomycetemcomitans* inoculation, and a small but significant decrease in monocyte count compared with non-infected rats ($p < 0.05$).

Aggregatibacter actinomycetemcomitans infection enhances apoptosis in vivo through a caspase-3-dependent mechanism in experimental periodontitis.

Goto-kakizaki (GK) and Wistar rats were inoculated with *A. actinomycetemcomitans.* GK develops type 2 diabetes approximately at age 8 weeks. A total of 38 diabetic rats and 33 normal rats were used for the study and euthanized at baseline and at 4, 5, and 6 weeks after inoculation (Appendix 1-4).

Antibody titers were measured to evaluate if *A. actinomycetemcomitans* infected the animals. Diabetic rats presented high numbers of antibody 6 weeks after the infection ($p < 0.05$). They had a 2-fold increase in antibody titer expression compared with the infected normal rats ($p < 0.05$).

The impact of *A. actinomycetemcomitans* infection on total anaerobic bacteria levels in noninfected *vs* infected normoglycemic and diabetic rats did not show any significant difference.

Lymphocytes population was measured. Normal and diabetic rats did not show any difference before and after *A. actinomycetemcomitans* infection. However, when comparing both groups, lymphocytes in the peripheral circulation diabetic infected rats presented fewer lymphocytes and more granulocytes compared to infected normoglycemic rats ($p < 0.05$).

Bone loss was increased in diabetic rats after the infection. When compared to normal rats, the distance from the cement-enamel junction (CEJ) to the bone crest in diabetic rats was 1.7-fold greater than in normal rats ($p < 0.05$).

Moreover, PMNs levels in noninfected diabetic and normal rats were low. After *A. actinomycetemcomitans* infection, PMNs increased in the gingival epithelium 5.3-fold in diabetic rats and 3.4-fold in normal rats ($p < 0.05$).

TNF-α positive cells and apoptotic cells were significantly higher in gingival epithelium and connective tissue in diabetic rats after the infection when compared to normal rats ($p < 0.05$).

Antibiotic treatment significantly decreased antibody titers, PMNs infiltration, bone loss and TNF-α in the diabetic rats ($p < 0.05$) while it remain equal in the normal animals. Apoptosis was not diminished by the use of an antibiotic in diabetic rats. However, the treatment with the inhibition of caspase-3/7 plus antibiotic did reduce the number of apoptotic cells in the gingival epithelium and gingival connective tissue by reversing the impact of *A. actinomycetemcomitans* infection to baseline levels in diabetic rats ($p < 0.05$).

Diabetes aggravates periodontitis by limiting repair through enhanced inflammation.

Goto-Kakizaki (GK) and normoglycemic Wistar rats were used for this study (Appendix 1-3). Periodontitis was induced by the placement of a ligature around the maxillary second molars facilitating bacterial invasion of the gingiva. Rats were euthanized on day 0, 7 (ligature removal), 11, 15 and 22 (Appendix 5).

Periodontal bone resorption has been related to the transition of the inflammatory infiltrate from the area below to the epithelium to the area of connective tissue close to the bone. At baseline PMNs and mononuclear leukocytes in both normoglycemic and diabetic groups were found in close proximity to the epithelium. The inflammatory infiltrate moved deeper along with the connective tissue in both groups within the onset of periodontitis.

The number of osteoclast was low in both normoglycemic and diabetics rats and increased significantly at the initiation of periodontal disease.

At baseline TNF-α, IFN-γ and IL-1β mRNA expression in the diabetic group was also low.

The amount of new bone formation, osteoid and the number of osteoblasts was low in normoglycemic and diabetic groups at baseline and during the initiation of periodontal inflammation.

On day 0 the number of apoptotic cells was also low but increased 12-fold on day 7 in both normoglycemic and diabetic groups ($p<0.05$).

Furthermore, proliferation of bone-lining cells (PCNA), FGF-2 and BMP-2 positive cells was low in both groups at baseline and during the initiation of periodontal inflammation.

TGF β -1, BMP-2, BMP-6 and FGF-2 mRNA levels were low at baseline.

Once periodontal disease ceased, the depth of the infiltrate was reduced in the normoglycemic group while it remained substantially higher in the diabetic group ($p<0.05$). TNF-inhibitor decreased the levels of the inflammatory infiltrate.

TNF-α, IFN-γ and IL-1β l mRNA levels in the diabetic group were higher initially after removal of ligatures and declined closer to baseline levels by day 15. Inhibition of TNF decreased the levels of these above-mentioned cytokines.

Within 4 days of removal of ligatures new osteoid formation increased 4-fold and new bone formation increased 10-fold in the normal group peaking 8 days after ligatures were removed ($p<0.05$). The diabetic group did not have a significant burst of osteoid or bone formation. Number of osteoblasts in the normoglycemic group increased 2.7-fold during the resolution of inflammation. The diabetic group had significantly fewer numbers of osteoblasts at each of this time points ($p<0.05$). When treated with a TNF inhibitor, new osteoid and bone formation and the number of osteoblasts increased to a level equivalent to that of the normoglycemic group.

After the ligature was removed the number of apoptotic cells in the normoglycemic group decreased substantially and continued to decrease with time. The diabetic group followed a similar pattern at each of these time points but it was 1.7 to 2-fold higher than the normoglycemic group and this higher level of apoptosis was blocked by the treatment with TNF inhibitor.

PCNA, FGF-2 and BMP-2 showed a significant difference between the diabetic and the normal group during the resolution period of the inflammation. The number of positive cells increased in the normoglycemic group after the ligature removal but there was relatively small change in the diabetic animals in both cases ($p<0.05$). After treated with a TNF inhibitor new bone formation, PCNA, FGF-2 and BMP-2 positive cells in the diabetic group was increased to a level equivalent or a little bit higher to that of the normoglycemic group ($p<0.05$).

During the resolution of inflammation diabetes growth factor mRNA levels were reduced, which was reversed by TNF inhibitor.

DISCUSSION

DISCUSSION

Localized aggressive periodontitis produces a great and fast destruction of periodontal tissues and is driven mainly by *A. actinomycetemcomitans* (36). It has previously been shown the effect of this bacterium on bone loss (37-39), but there are no studies showing neither the effect in a histologic and hematologic level, nor the effect of antibacterial treatment after *A. actinomycetemcomitans* infection.

Our study shows that *A. actinomycetemcomitans* infection stimulated the expression of PMNs and TNF-α both in the junctional and gingival epithelium. This expression drove to greater loss of attachment in the infected rats. These changes were reversed by the use of an antibiotic treatment, suggesting that TNF-α might suppress the activity of cells needed to produce attachment, or by the induction of lytic enzymes that breakdown proteins (40). The number of osteoclasts also increased after *A. actinomycetemcomitans* inoculation, and did not diminish even with the administration of antibiotic, likely because the period of observation was not long enough to exercise any effect. *A. actinomycetemcomitans* infection also stimulated the adaptive immune response by increasing the levels of peripheral blood monocytes and CD8+T cells. These cells were obtained from local draining cervical and submandibular lymph nodes of *A. actinomycetemcomitans*-infected rats and control rats. This adaptive immune response correlates with previous findings where gingival CD8+T cells were seen increased in gingivitis, periodontal disease and aggressive periodontitis (41-43).

Moreover, of the Wistar rats exposed to *A. actinomycetemcomitans*, 78% became infected. In previous studies, other models of rats were evaluated, and showed infection rate to be 17% to 83%, depending on the rat strain. The model we used was previously examined within 12 weeks time point (37, 38). The present study examined the rats within 4 weeks. To increase the level of infection the animals received the inoculum by oral gavage during the first week of feeding protocol. Diabetic rats showed a greater local response with higher level of TNF-α and PMNs compared to Wild type rats after *A. actinomycetemcomitans* infection. Elevated levels of antibody against *A. actinomycetemcomitans* in diabetic rats were found, as compared to Wild-type rats. The number of lymphocyte population collected from blood was found to be lower in diabetic

rats compared to Wild-type. All this may be explained because cytolethal distending toxin (CDT) underwent apoptosis or possibly because of an increase in granulocytes. Higher local response seen in this study and in prior studies is correlated to greater bone loss in the diabetic rats.

This greater bone loss may be due in part to an increased apoptosis. This study is the first *in vivo* demonstration that apoptosis is increased after *A. actinomycetemcomitans* infection in diabetic animals. The mechanism through which the apoptosis seems to work is caspase-3/7. When caspase-3/7 was inhibited the apoptosis was reversed in gingival epithelium and connective tissue rats. It is possible that CDT and the increased TNF-α levels presented in diabetic rats may help explain the mechanisms by which this apoptosis occurs (44, 45)

Antibiotic treatment has been used as a complementary therapy to treat localized aggressive periodontitis (46-48). The impact of antibiotic treatment on different parameters was assessed. For *A. actinomycetemcomitans* antibody titer, PMNs infiltration, TNF-α levels, and apoptosis, only the diabetic rats were affected by the antibiotics. This may indicate that diabetic rats have an incapacity to fight against the bacteria that contributes to greater proinflammatory events.

Furthermore, the present study demonstrates that diabetes affects the resolution phase of the inflammation by limiting new bone formation. Bone formation is linked to the level of inflammation in the diabetic periodontium since the amount of new bone increased when TNF was inhibited. Inhibition of TNF also caused a general reduction in cytokine levels associated with both innate and adaptative immune response. This prolonged inflammatory response caused a prolonged period of high osteoclast numbers, which was reversed with pegsunrecept. Thus, a consequence of prolonged inflammation in the diabetic periodontum poses difficulty in turning off osteoclasts and bone resorption, leading to a longer period of periodontal destruction.

The production of growth factors that control proliferation and apoptosis were significantly decreased when inflammation was inhibited. These results provide a mechanistic explanation for diminished bone formation in diabetic animals due to the

negative impact of inflammation on growth factors that stimulate proliferation and inhibit apoptosis.

Thus, the infection by *A. actinomycetemcomitans* bacteria in rats shows to be a good model to study the onset, progression and mechanisms that are involved in periodontal disease. Moreover, diabetes aggravates the progression and resolution of periodontal disease affecting apoptosis and other mechanisms such as grow factors that are involved in bone coupling.

CONCLUSION

CONCLUSION

1. **Evaluate if *A. actinomycetemcomitans* is a good model to study the onset and progression of inflammation in periodontal disease and how is modified by antibiotic treatment.**

In conclusion, it has been demonstrated that Wistar rats are a good model to observe the effects of inflammation of *A. actinomycetemcomitans*-induced periodontal disease.

A. actinomycetemcomitans infection increases the levels of CD8+T cells in the lymph nodes and peripheral blood monocytes. *A. actinomycetemcomitans* infection induces the expression of TNF-α in the junctional epithelium, increases osteoclasts numbers and causes loss of attachment. This attachment loss was reversed by the use of antibiotic treatment giving us more insight into the pathways of periodontal disease (Hypothesis 1).

2. **Evaluate how diabetes modifies the onset and progression of inflammation in periodontal disease and how is modified by antibiotic treatment.**

Additionally, diabetes increased inflammatory response to *A. actinomycetemcomitans* with excessive TNF-α expression in periodontal tissue and enhanced alveolar bone resorption. The latter maybe affected by increased apoptosis in periodontal tissue as it was shown with the increased number of apoptosis in gingival epithelium and connective tissue rats (Hypothesis 2). The mechanism through this apoptosis seems to occur is caspase-3, due that the blocking caspase-3 showed to reverse apoptosis in gingival epithelium and connective tissue rats (Hypothesis 3).

Antibiotics were able to reverse many parameters of the local host response in diabetic animals but not in the normoglycemic animals. This might be due to a deficit in the capacity of diabetic animals to resist infection. This information provides valuable insight as to how diabetes may alter host-bacterium interactions in a way that promotes periodontal breakdown (Hypothesis 4).

3. **Look at the resolution of the inflammation and how this affects in bone coupling.**

Moreover, we examined the effect of diabetes during the resolution of periodontal inflammation and found that diabetes prolongs enhanced inflammation. Diabetic rats had reduced bone coupling compared to the normoglycemic group. This finding was linked to inflammation, as inhibiting TNF reversed the amounts of osteoblasts cells, new bone and osteoid (Hypothesis 5). The mechanisms by which inflammation affects bone were studied by examining factors that regulate bone cells. These factors were limited by the production of growth factors that control proliferation and apoptosis. When TNF-α was inhibited improved the behavior of the diabetic model similar to the normoglycemic rats (Hypothesis 3).

These results provide an explanation of how diabetes can negatively affect bone through the effects of enhanced inflammation on the expression of critical factors needed to stimulate bone formation.

PUBLISHED ARTICLES

J Clin Periodontol 2012; 39: 333–341 doi: 10.1111/j.1600-051X.2011.01847.x

Journal of Clinical Periodontology

A.actinomycetemcomitans-induced periodontal disease promotes systemic and local responses in rat periodontium

Beatriz de Brito Bezerra[1], Oelisoa Andriankaja[2], Jun Kang[2,3], Sandra Pacios[2,5], Hyung Jin Bae[2], Yu Li[4], Vincent Tsiagbe[4], Helen Schreiner[4], Daniel H. Fine[4] and Dana T. Graves[2]

[1]Department of Prosthodontics and Periodontics, Piracicaba Dental School, University of Campinas, Piracicaba, São Paulo, Brazil; [2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA; [3]Department of Periodontology, School and Hospital of Stomatology, Peking University, Beijing, China; [4]Department of Oral Biology, New Jersey Dental School, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA; [5]Department of Periodontology, School of Dental Medicine, Universitat Internacional de Catalunya, Sant Cugat del Vallès, Spain

de Brito Bezerra B, Andriankaja O, Kang J, Pacios S, Bae HJ, Li Y, Tsiagbe V, Schreiner H, Fine DH, Graves DT. A.actinomycetemcomitans-*induced periodontal disease promotes systemic and local responses in rat periodontium. J Clin Periodontol 2012; 39: 333–341. doi: 10.1111/j.1600-051X.2011.01847.x.*

Abstract

Aim: To characterize the histologic and cellular response to *A. actinomycetemcomitans* (*Aa*) infection.

Material and Methods: Wistar rats infected with *Aa* were evaluated for antibody response, oral *Aa* colonization, loss of attachment, PMN recruitment, TNF-α in the junctional epithelium and connective tissue, osteoclasts and adaptive immune response in local lymph nodes at baseline and 4, 5 or 6 weeks after infection. Some groups were given antibacterial treatment at 4 weeks.

Results: An antibody response against *Aa* occurred within 4 weeks of infection, and 78% of inoculated rats had detectable *Aa* in the oral cavity ($p < 0.05$). *Aa* infection significantly increased loss of attachment that was reversed by antibacterial treatment ($p < 0.05$). TNF-α expression in the junctional epithelium followed the same pattern. *Aa* stimulated high osteoclast formation and TNF-α expression in the connective tissue ($p < 0.05$). PMN recruitment significantly increased after *Aa* infection ($p < 0.05$). *Aa* also increased the number of $CD8^+$ T cells ($p < 0.05$), but not $CD4^+$ T cells or regulatory T cells (Tregs) ($p > 0.05$).

Conclusion: *Aa* infection stimulated a local response that increased numbers of PMNs and TNF-α expression in the junctional epithelium and loss of attachment. Both TNF-α expression in JE and loss of attachment was reversed by antibiotic treatment. *Aa* infection also increased TNF-α in the connective tissue, osteoclast numbers and $CD8^+$ T cells in lymph nodes. The results link *Aa* infection with important characteristics of periodontal destruction.

Key words: *A.actinomycetemcomitans*; animals; bone resorption; disease models; host-pathogen interactions; periodontal disease

Accepted for publication 12 December 2011

Conflict of interest and source of funding statement

The authors declare they have no conflict of interests. This study was supported by grants from the NIDCR (DE017732 and DE018307). Author BBB was the recipient of a scholarship from the CAPES Foundation in Brazil.

Localized aggressive periodontitis (LAgP), previously known as localized juvenile periodontitis, is characterized by relatively rapid and severe destruction of periodontal tissues affecting the first molars and central incisors, which is usually detected and diagnosed during puberty in systemically healthy individuals (Armitage & Cullinan 2010). This form of periodontitis presents minimal signs of clinical inflammation and plaque build-up (Armitage & Cullinan 2010). It is more frequently seen in patients of African descent (Löe & Brown 1991, Brown et al. 1996, Fine et al. 2007) and is usually found in other family members (Tonetti & Mombelli 1999, van Winkelhoff & Boutaga 2005). The primary

suspected pathogen associated with this form of periodontal disease is *A. actinomycetemcomitans* (*Aa*).

Aa is a gram-negative non-motile bacterium that is indigenous to the oral cavity (Henderson et al. 2010) which is also associated with endocarditis (Paturel et al. 2004). Many studies have shown high levels of *Aa* in samples obtained from patients with localized aggressive periodontitis compared with periodontally healthy, gingivitis and patients with other forms of periodontal disease (Slots et al. 1980, Hamlet et al. 2001, Suda et al. 2003, Haubek et al. 2008). After periodontal treatment, a reduction in *Aa* is consistent with clinical improvement (Christersson et al. 1985, Mandell & Socransky 1988, Takamatsu et al. 1999), and the presence of *Aa* can be an indicative of future disease progression (Fine et al. 2007). It is estimated that 90% of patients were diagnosed with LAgP, and 10–20% healthy patients harbour *Aa* (Hamlet et al. 2001, Darout et al. 2002, Suda et al. 2003). The oral mucosa has been identified as an initial colonization site and primary reservoir (Rudney et al. 2005). *Aa* is also able to colonize tooth surfaces and the subgingival space, where it may lead to disease progression (Fine et al. 2005, 2007, Rudney et al. 2005).

Recently, a new animal model has been developed to study the effects of *Aa* colonization on periodontal disease progression (Fine et al. 2001, Schreiner et al. 2003, 2011, Li et al. 2010). This model consists of infecting rats with the rough strain of *Aa*, known to adhere to different surfaces, including the buccal epithelial and teeth (Fine et al. 2001, Fine & Furgang 2002). This model has provided knowledge on how the adaptive immune response is regulated (Li et al. 2010). It is also well suited to examine aspects of bacterial behaviour that promote colonization and initiation of periodontal disease (Graves et al. 2008). Previous studies have utilized different strains of rats, and more recently, it has been established that different patterns of infection and bone loss can be found between rat strains (Schreiner et al. 2011). However, the local impact of *Aa* in this animal model has not previously been examined at the histological or cellular level. Thus, the aim of this study was to characterize the local and systemic response to the *Aa* infection in the Wistar rat to better understand the consequences of periodontal bone loss due to bacterial challenge by analysing the response at the histological and cellular level.

Material and Methods

Animals

The rat model has been previously described (Schreiner et al. 2003). Briefly, 44 Wistar rats (16–20 weeks of age) were purchased from Charles River (Wilmington, MA), housed in separate cages and fed powdered food (Laboratory Rodent Meal Diet 5001; Purina Mills Feeds, St. Louis, MO, USA). To depress the 'natural' resident flora, rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days. During the last 2 days of antibiotic treatment, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex; Procter and Gamble, Cincinnati, OH, USA). After a subsequent period of 3 days without antibiotic treatment, the rats were divided into five groups of approximately seven rats each. The adherent *Aggregatibacter actinomycetemcomitans* (*Aa*) strain, Columbia University *Aa* clinical isolate #1000 (CU1000NRif) (*N* = nalidixic acid resistant; rif = rifampicin resistant) was grown in *Aa* growth medium containing rifampicin (35 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) as described (Schreiner et al. 2011) for 2 days at 37°C in a 10% CO_2/90% air atmosphere. After fasting for 3 h, rats received 10^8 *Aa* cells in 1 g of powdered food supplemented with 3% sucrose in PBS placed in special feeder trays (1 ml). This protocol was followed for 8 days (Schreiner et al. 2003). During the first 4 days of the feeding protocol, the animals also received 10^8 *Aa* cells in 1 ml of inoculation solution by oral gavage. After 1 h, the inoculated food was removed and replaced with regular powdered food. After the feeding protocol was over, the animals were euthanized 4, 5 and 6 weeks later. Baseline animals did not undergo antibacterial treatment and were not inoculated with *Aa*, but did receive powered food supplemented with 3% sucrose under the same conditions as experimental rats. The study was reviewed and approved by the Institutional Animal Care and Use Committee.

Antibacterial treatment post-feeding

Four weeks after *Aa* inoculation, two groups of animals, with approximately seven rats each, received a daily dose of kanamycin (20 mg) and ampicillin (20 mg) in the water for 4 days with the intention to stop the infection. Concomitantly, the oral cavities of these rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex; Procter and Gamble). These animals were euthanized 1 and 2 weeks after the start of the antibacterial regimen.

Sampling of rat oral microflora

Two microbial samples were collected, one after the inoculation of *Aa* and the other at the time of euthanasia. The rats were anaesthetized, and their oral microflora was sampled with a cotton tip swab for soft tissue sampling and a toothpick (Johnson & Johnson, Piscataway, NJ, USA) for hard tissue sampling. Both samples were combined in tubes containing 1 ml PBS. Serial tenfold dilutions were made and plated on trypticase soy agar (TSA) with 5% sheep blood (BD Biosciences, San Jose, CA, USA) for total anaerobe counts. Trypticase soy agar plates were incubated in an anaerobic atmosphere at 37°C for 7 days to obtain total bacterial counts. To determine whether *Aa* colonized the oral cavities of inoculated rats, bacterial DNA was isolated from the collected oral samples with a DNA extraction kit (Qiagen, Valencia, CA, USA) and subjected to PCR analysis. Forward and reverse primers (5′-GGAATTCCTAGGTATTGCGAAACAATTTGATC-3′ and 5′-GGAATTCCTGAAATTAAGCTGGTAATC-3′, respectively) amplified a 262-base-pair PCR product from the *Aa* leukotoxin gene as previously described (Goncharoff et al. 1993).

Level of antibody to *Aa*

IgG antibody reactive with *Aa* was assessed using enzyme-linked immunosorbent assay (ELISA). Blood was collected by cardiac puncture, and

serum was obtained and stored at −20°C. An *Aa* lysate was prepared and used to coat the wells of microtitre dishes (NUNC-ImmunoPlate with Maxi Sorp surface; Thermo Fisher Scientific, Rochester, NY, USA). A standard curve was generated using purified rat IgG (Sigma-Aldrich) in carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich). Rat serum diluted 1/5 and 1/10 in blocking buffer was added to the wells coated with the *Aa* pellet lysate. The serum dilutions were added, in duplicate. The microtitre plates were covered and incubated for 2 h at room temperature, and the wells were washed, incubated with rabbit anti-rat IgG-Fc conjugated to alkaline phosphatase (Bethyl Laboratories; Montgomery, TX, USA) and quantified using p-nitrophenyl phosphate substrate (Sigma-Aldrich). After incubation for 1 h, the absorbance of the microplate wells was read on a microplate reader at 405 nm.

Rats were considered to have been positive for *Aa* infection if PCR results showed the *Aa* leukotoxin band or if antibody levels were equal to or greater than twice the mean baseline levels. Animals were considered to be not-infected even if they had been exposed to *Aa*, and if both PCR results for *Aa* leukotoxin and antibody levels were negative. For time course analysis, only infected animals were included.

Histomorphometry

Right maxillae were collected at euthanasia, hemi-sected and fixed in 4% paraformaldehyde in PBS for 48 h at 4°C with the solution changed twice a day. After 48 h, specimens were washed with PBS and placed in 10% EDTA (pH 7.0) for decalcification. Paraffin-embedded sagittal sections were prepared at a thickness of 5 microns, and the interproximal areas between the 1st and 2nd and the 2nd and 3rd maxillary molars were examined. The mid-interproximal region was examined in each specimen and was established by sections where the root canal systems were clearly visible. Two randomly chosen sections of each interproximal area were examined at 200× magnification. All data were analysed by a blinded examiner who did not know the group to which an animal belonged. The examiner was calibrated against a second individual. Attachment loss was measured by the distance between the cemento-enamel junction and the most coronal extent of connective tissue attachment to cementum. Millimetre was the measurement unit selected for this analysis. Local inflammatory response in the gingival epithelium was evaluated by counting the number of polymorphonuclear (PMN) cells per area at 600× magnification.

Osteoclasts

Osteoclasts were recognized as positive tartate resistant acid phosphatase (TRAP) staining on large multinucleated cells directly lining the bone surface in Howship's lacunae. Osteoclast numbers were determined by quantifying number of osteoclasts per mm of bone surface. As rat molars undergo distal drift (Moss-Salentijn & Moss 1977), there is constant resorptive activity on the proximal bone between the molars. Therefore, the analysis of TRAP-stained sections was restricted to the distal aspect of the interproximal bone. After quantification of osteoclasts, two categories were determined. Animals were categorized as having high osteoclast numbers, if the number of osteoclasts was ≥ 2, and low osteoclast number, if there was 1 or 0 osteoclasts present. Percentage of high and low osteoclast numbers were calculated for statistical analysis.

TNF-α

To evaluate cells that expressed TNF-α in the gingiva epithelium or junctional epithelium (JE), sections were examined using immunohistochemistry with an antibody against TNF-α (IHCWORLD; Woodstock, MD, USA) and examined at 600× magnification. Immunoreactivity was judged by the following scale: 0, no positive cells; 1, three to four positive cells per field with weak immunostaining; 2, four to ten positive cells per field with strong immunostaining; and 3, more than 10 positive cells per field with strong immunostaining.

Systemic leucocyte response

Rat lymph node cells were prepared and analysed as described (Li et al. 2010). Briefly, at day 0 and after animals had been infected with *Aa* (4, 5 or 6 weeks), single-cell suspensions were obtained from the submandibular and cervical lymph nodes of the rats. Lymphocyte populations were isolated using Ficoll–Hypaque density gradient centrifugation. Flow cytometry was conducted on unfractionated lymphocytes. FlowJo software (Tree Star; Inc., Ashland, OR, USA) was used to analyse the fluorescence-activated cell sorting (FACS) data. Anti-CD32 (clone D34-485), for blocking Fcγ II receptor, was purchased from BD Biosciences. Anti-IA [major histocompatibility complex (MHC) class II, clone 14-4-4S] antibody obtained from American Type Culture Collection (Manassas, VA, USA) was fluorescein isothiocyanate (FITC)-conjugated by us. FITC labelled anti-CD4 (clone OX35), anti-CD3 (clone G4.18) and PE-labelled anti-CD4 (clone OX38) were purchased from BD Biosciences. FITC-labelled anti-FoxP3 (clone FJK-16s) and PE-labelled anti-CD25 (clone OX39) were purchased from eBioscience (San Diego; CA, USA). For haematologic analysis, 1 ml of blood was collected from each rat at time of euthanasia into tubes with EDTA (BD Biosciences). Blood was analysed using HemaTrue Hematology Analyzer (HESKA, Loveland, CO, USA).

Statistical analysis

Fisher's exact test was applied to compare the percentage of animals that were positive or negative for *Aa* in baseline and after exposure to *Aa*. The same statistical test was applied to compare specimens with high osteoclast numbers between baseline and 4, 5 and 6 weeks after *Aa* infection. Pearson's chi-squared test was applied to compare the percentage of animals with a positive or negative antibody threshold to *Aa* before or after the second antibacterial treatment. This same test was also applied to compare osteoclast numbers between rats with or without antibacterial treatment following *Aa* infection. The Mann–Whitney test was applied to compare antibody titres against *Aa* in animals treated or not with antibiotics plus chlorhexidine after *Aa* infection. Median values, instead of mean values, were used for the analysis of total

anaerobe counts, as outlier values were left in the data set. The median test was then applied to compare total anaerobe counts at baseline to 4, 5 and 6 week time points, *Aa*-infected and non-infected rats, as well as in rats with or without antibacterial treatment following *Aa* infection. A two-tailed T-test was used to compare lymphocyte populations and leucocyte populations from peripheral blood between *Aa*-infected and non-infected rats; LOA, PMNs and levels of TNF-α in junctional epithelium and gingiva between *Aa*-infected and non-infected rats and animals treated or not with antibiotics plus chlorhexidine after *Aa* infection. A one-way ANOVA test with contrast was used to compare LOA and PMNs between baseline and 4, 5 and 6 week infection time points. Significance levels were set at 5%.

Results

Aa colonization

To establish whether animals exposed to *Aa* were infected, samples were assessed from soft and hard tissue of the oral cavities at the time of euthanasia. The bacterial colonies tested positive for *Aa* using leukotoxin-specific PCR. No *Aa* was observed in rats not inoculated with *Aa*. Following inoculation, 78% of the rats had detectable *Aa* in the oral cavity using PCR at the 4 week time point compared with no animals at baseline ($p < 0.05$) (Fig. 1a).

Antibody titre and threshold

Antibody levels against *Aa* were assessed in pre- and post-infected animals. Four weeks after *Aa* inoculation, over 80% of the rats had positive antibody titres defined as values twofold higher than baseline ($p < 0.05$) (Fig. 1b). When comparing animals that received antibacterial treatment to those without such treatment, no change was observed in per cent antibody threshold levels (Fig. 1c) or antibody titre ($p > 0.05$) (Fig. 1d).

Total anaerobe bacteria counts

Using samples collected from the oral cavity, total anaerobe bacterial counts were evaluated before and after *Aa* inoculation (Fig. 2a–c). No statistical significant differences were observed in any of the evaluated comparisons ($p > 0.05$).

Loss of attachment after *Aa* inoculation

Loss of attachment (LOA) was measured histologically. Following

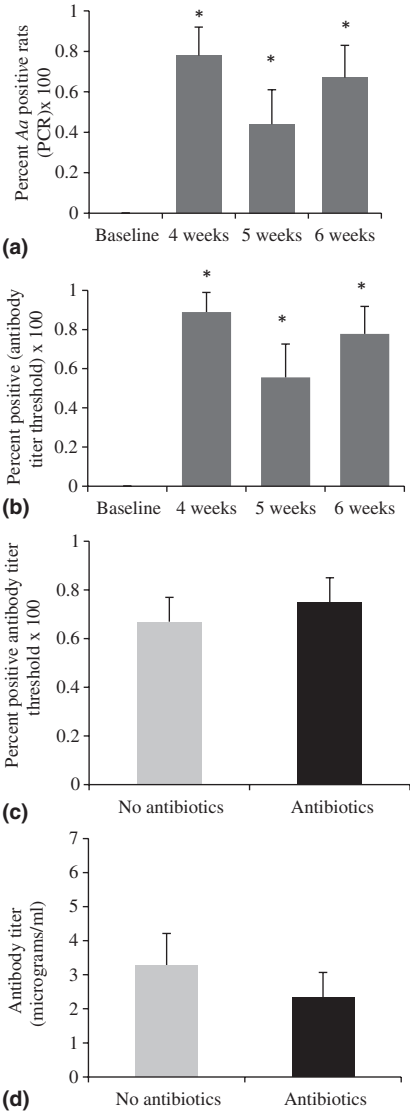


Fig. 1. Aa infection and *A*nti-*Aa* antibody levels. (a) Swabs were obtained from the oral epithelium and tooth surface and examined for the presence of *Aa* by PCR. The per cent of *Aa* positive animals following inoculation at baseline and at 4, 5 and 6 weeks post-inoculation. Significance was determined using Fisher's exact test (b). The per cent rats with anti-*Aa* IgG levels at least twofold higher than the mean baseline level at baseline or 4, 5 and 6 weeks after inoculation was determined using ELISA. Significance was determined using Fisher's exact test. (c) Per cent rats with antibody titres at least twofold higher than the mean baseline level with or without antibiotic plus chlorhexidine treatment following *Aa* inoculation. Significance was determined using the Pearson chi-square test. (d) Anti-*Aa* IgG levels in serum collected from *Aa* inoculated animals with or without anti-bacterial treatment. Significance was determined using the Mann–Whitney test. The data are represented as mean ± SEM. *$p < 0.05$.

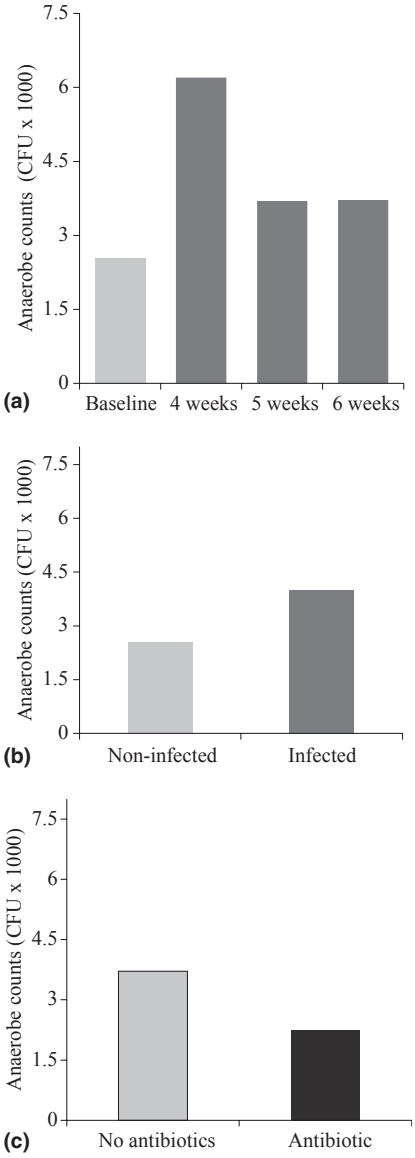


Fig. 2. Total anaerobe counts. Total anaerobe counts were obtained prior to and at different time points after infection with *Aa*. (a) Number of CFU at baseline and following inoculation with *Aa*. (b) Total anaerobe counts from not-infected and *Aa*-infected rats. (c) Total anaerobe counts from *Aa*-fed animals with antibacterial treatment ($p > 0.05$). Data are presented as median; the median test was used for statistical significance. None of the values were significant ($p > 0.05$).

inoculation with *Aa*, a significant LOA was observed in all experimental time points compared with baseline ($p < 0.05$) (Fig. 3a, Figure S1). When considering *Aa* infected animals as a group, a sixfold increase in LOA was found ($p < 0.05$) (Fig. 3b). Antibacterial treatment promoted a reduction in LOA compared with the untreated group ($p < 0.05$) (Fig. 3c).

Osteoclast numbers post *Aa* infection

The impact of *Aa* infection on osteoclast formation was examined as the per cent rats with high osteoclast numbers, which had the highest levels at 6 weeks following *Aa* infection ($p < 0.05$) (Fig. 4a, Figure S2). As a group, 52% of animals had high numbers of osteoclasts after *Aa* inoculation compared with none in animals that had not been infected with *Aa* ($p < 0.05$) (Fig. 4b). Antibacterial treatment did not affect the per cent of rats that had high numbers of osteoclasts compared with untreated animals in the time frame tested (Fig. 4c).

Inflammatory response to *Aa* infection

Local inflammatory response was evaluated both by counting the number of PMNs in the gingival epithelium as well as by TNF-α expression in the gingival epithelium and JE. PMN numbers increased at threefold to fourfold after *Aa* infection ($p < 0.01$) (Fig. 5a, Figure S3), whereas the 6 week time point showed no significant difference compared with baseline ($p > 0.05$). When considering *Aa* infected animals as a group, there was a 3.4-fold

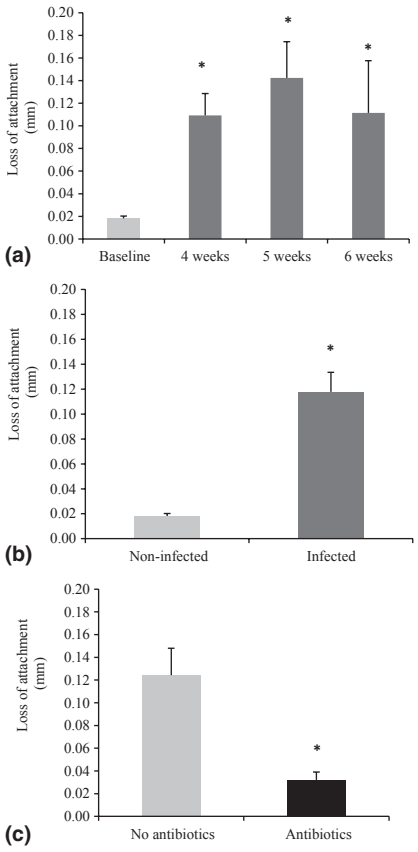


Fig. 3. Loss of attachment. Loss of attachment (LOA) was measured by the distance between the cemento-enamel junction and the most coronal extent of connective tissue attachment to cementum. (a) LOA in not-infected (baseline) animals and *Aa*-infected animals at 4, 5 and 6 weeks. Significance was determined using one-way ANOVA. (b) LOA in not-infected and *Aa*-infected animals. Significance was determined using Student's *t*-test. (c) LOA in *Aa*-infected animals treated or not with antibiotics plus chlorhexidine. Significance was determined using Student's *t*-test. Data are presented as mean ± SEM. $*p < 0.05$.

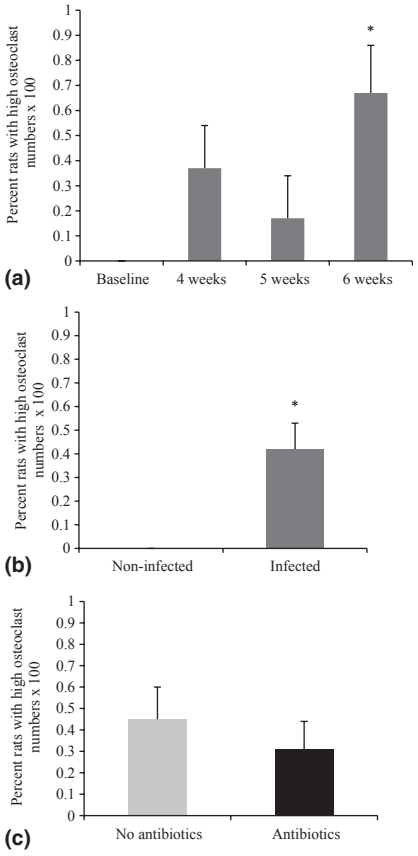


Fig. 4. Osteoclasts. Osteoclasts were recognized as TRAP positive, multinucleated cells directly lining the bone surface. (a) Per cent rats with high osteoclasts numbers in not-infected (baseline) animals and *Aa*-infected animals at 4, 5 and 6 weeks. Significance was determined using Fisher's exact test. (b) Per cent rats with high osteoclast numbers in not-infected and *Aa*-infected animals. Significance was determined using Fisher's exact test. (c) Per cent rats with high osteoclast numbers in *Aa*-infected animals with or without antibacterial treatment. Significance was determined using Chi-square test. $*p < 0.05$.

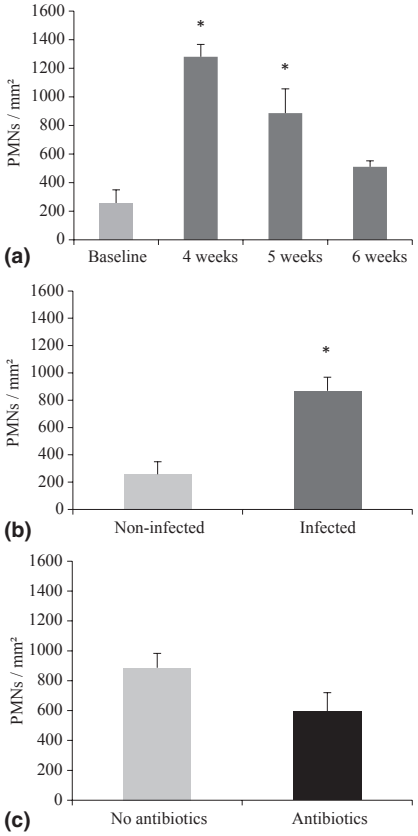


Fig. 5. Polymorphonuclear (PMNs) leucocytes in gingival epithelium. PMNs were counted in HE stained sections at 1000 × magnification and presented as number of PMNs per mm^2. (a) Number of PMNs/mm^2 in non-infected (baseline) animals versus *Aa*-infected animals at 4, 5 and 6 weeks. Significance was determined using One-way ANOVA test. (b) Number of PMNs/mm^2 in non-infected and *Aa*-infected animals. Significance determined using Student's *t*-test. (c) Number of PMNs/mm^2 in *Aa*-infected animals treated or not with antibiotics plus chlorhexidine. Significance determined using Student's *t*-test. Data are presented as mean ± SEM. $*p < 0.05$.

increase in the number of PMNs/mm^2 after infection ($p < 0.004$) (Fig. 5b). Antibiotics administration post-infection did not significantly reduce the number of PMNs in the epithelium ($p > 0.05$) (Fig. 5c).

When *Aa* inoculated animals were examined as group, there was a 1.6-fold increase in TNF- α positive cells in the JE compared with not-infected animals ($p < 0.05$) (Fig. 6a, Figure S4). A similar increase was found in the gingiva of infected animals (1.5-fold) ($p < 0.05$) (Fig. 6b). Antibacterial treatment promoted a significant 25% decrease in the number of cells producing TNF- α in the JE ($p < 0.05$) (Fig. 6c). In contrast, antibacterial treatment did not affect the number of TNF-α expressing cells in the gingiva (Fig. 6d).

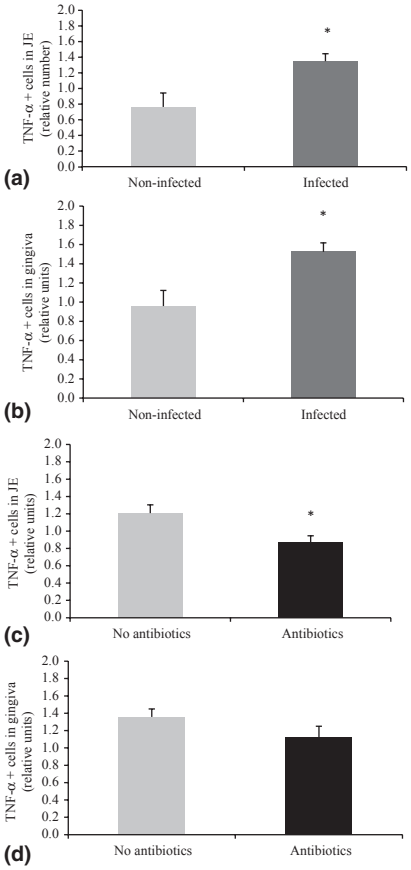


Fig. 6. TNF-α positive cells in junctional epithelium and gingival connective tissue. TNF-α immunopositive cells were examined using immunohistochemistry and assessed using the following scale at 600× magnification: 0, no positive cells; 1, three to four positive cells per field with weak immunostaining; 2, four to ten positive cells per field with strong immunostaining; and 3, more than 10 positive cells per field with strong immunostaining. (a) TNF-α positive cells in JE in non-infected and *Aa*-infected animals. (b) TNF-α positive cells in JE in *Aa*-infected animals treated or not with antibiotics plus chlorhexidine. (c) TNF-α positive cells in gingival connective tissue of non-infected and *Aa*-infected animals. (d) Number of TNF- α positive cells in gingival connective tissue of *Aa*-infected animals treated or not with antibiotics plus chlorhexidine. Data are presented as mean ± SEM. Significance was determined using Student's *t*-test. *$p < 0.05$.

Systemic response

Leucocyte populations in lymph nodes and blood were examined as a measure of the systemic response. No significant differences were observed in B cell activation (MHC II) and CD4$^+$ T cell numbers in the infected animals compared with non-infected animals (Table 1). However, CD8$^+$ T cell numbers showed a 1.6-fold increase after *Aa* inoculation that was significant ($p < 0.05$). No change in activation of regulatory T (Tregs) cells (CD4$^+$CD25$^+$ and CD4$^+$FoxP3$^+$) was observed in infected animals compared with non-infected ($p > 0.05$). Blood collected at time of euthanasia was analysed for major leucocyte populations (Table 2). Total white blood cells (WBC), lymphocytes and granulocytes or their percentage did not change after *Aa* infection (Table 2). Monocyte number and per cent exhibited a small but significant decrease compared with non-infected rats ($p < 0.05$).

Discussion

Localized aggressive periodontitis is known for its rapid and severe destruction of periodontal tissues, and is thought to involve infection by *Aa* (Schenkein et al. 2007, Armitage & Cullinan 2010). Studies evaluating a periodontal disease rat model with *Aa* inoculated in the food have shown that *Aa* infection reliably produces bone loss (Schreiner et al. 2003, 2011, Li et al. 2010). However, local histologic changes and haematologic examination of peripheral lymphoid compartments have neither been evaluated in this model, nor has the effect of antibacterial treatment after infection been examined.

In the present study, we evaluated several parameters of the local host response in the rat periodontium to *Aa* infection. Loss of attachment after *Aa* inoculation was evident within 4 weeks, and was six times greater than loss of attachment in non-infected animals. Loss of attachment was also reversed following antibacterial treatment, suggesting that attachment levels in the short term can experience both loss and gain. The number of PMNs in the junctional and gingival epithelium increased with *Aa* infection. The number of TNF-α expressing cells also increased with *Aa* infection.

To examine the effect of reversing the infection, we examined TNF-α expression in the JE and LOA after antibiotic treatment. It is striking that treatment with antibiotic resulted in a significant decrease in the number of epithelial cells expressing TNF-α, which coincided with a gain in attachment. Thus, changes in attachment levels may reflect the expression of inflammatory mediators such as TNF-α in the junctional epithelium. TNF-α could potentially prevent reattachment by suppressing the activity of cells needed to produce attachment or by the induction of lytic enzymes that breakdown proteins involved (Smith et al. 2009). Studies have shown that increased levels of TNF-α in the gingival crevicular fluid and periodontal

Table 1. Lymphocyte populations obtained from draining cervical and submandibular lymph nodes of *Aa*-infected and non-infected rats

	MHC II (% cells)	CD4$^+$ (% cells)	CD8$^+$ (% cells)	CD4$^+$CD25$^+$ (% cells)	CD4$^+$FoxP3$^+$ (% cells)
Non-infected ($n = 4$)	51 ± 4.5	36.1 ± 4.8	22.6 ± 4.1	5.2 ± 0.5	4.3 ± 0.5
Infected ($n = 9$)	48.3 ± 2.6	33.3 ± 1.9	36 ± 2	7.4 ± 0.7	4.6 ± 0.5
Fold difference	0.95	0.92	1.59*	1.42	1.07

MHC, major histocompatibility complex.
*$p < 0.01$, compared with non-infected rats.

Table 2. White blood cells from peripheral blood of *Aa*-infected and non-infected rats

	WBC (10^3 IU/ml)	LYMP (10^3 IU/ml)	MONO (10^3 IU/ml)	GRAN (10^3 IU/ml)	LYMP (%)	MONO (%)	GRAN (%)
Non-infected ($n = 5$)	7.9 ± 1.3	6.2 ± 1.1	0.4 ± 0.06	1.3 ± 0.2	77 ± 2.4	4.9 ± 0.5	18.1 ± 2.1
Infected ($n = 12$)	6.9 ± 0.7	5.2 ± 0.5	0.3 ± 0.03	1.4 ± 0.3	77.2 ± 3.3	3.3 ± 0.3	19.6 ± 3.1
Fold difference	0.87	0.84	0.75*	1.08	1.0	0.67*	1.08

WBC, white blood cells; LYMP, lymphocytes; MONO, monocytes; GRAN, granulocytes.
*$p = 0.02$, compared with non-infected rats.

tissues are correlated with periodontitis in humans (Cochran 2008, Graves 2008, Li et al. 2010).

The generation of osteoclasts was increased with *Aa* infection, but was not significantly impacted by antibacterial treatment. However, this may be due to the relatively short time frame examined after antibacterial treatment, 1–2 weeks.

In the study by Schreiner et al. (2011), different rat strains exhibited different rates of colonization by *Aa*. Some animals were more prone to infection than others, which ranged from 83% in Fawn Hooded Hypertensive rats to 33.3%, in Dahl Salt-sensitive rats, and 17% in Brown Norway rats. Wistar rats have not been previously evaluated in this model. We found that approximately 78% of Wistar rats exposed to *Aa* became infected as determined using PCR or cultural detection of *Aa* in bacterial samples. The results indicate that the Wistar rat strain is highly susceptible to *Aa* colonization using this infection model. Moreover, these rats developed positive antibody titres within 4 weeks of inoculation. In previous studies (Schreiner et al. 2003, 2011, Li et al. 2010), high antibody levels were observed within 12 weeks after inoculation, but earlier time points were not examined. It should be noted that in the present study, the animals also received the inoculum by oral gavage during the first week of the feeding protocol, providing a greater exposure to the pathogen compared with the original feeding protocol (Schreiner et al. 2003).

Oral bacteria can stimulate cells of the adaptive immune response by increased numbers of activated T- and B-lymphocytes in the periodontal tissues (Graves et al. 2008). We observed an increase in $CD8^+$ T cells in lymphocytes obtained from local draining cervical and submandibular lymph nodes of *Aa*-infected rats, compared with control rats. Gingival $CD8^+$ T cells have been implicated in gingivitis, chronic periodontitis and aggressive periodontitis (Sigusch et al. 2006, Artese et al. 2011, Lima et al. 2011). The fact that we observed an increase in $CD8^+$ T cells in local draining lymph nodes of *Aa*-fed rats, suggests an adaptive response to *Aa* that induced cytotoxic T cells. Changes in the per cent $CD4^+CD25^+$ T cells in lymph nodes from *Aa*-infected rats just missed significance compared with control rats ($p = 0.1$). $CD25^+CD4^+$ T cells expressing the FoxP3 transcription factor are regulatory T cells (Treg cells) that prevent a hyperactive adaptive immune response (Sakaguchi 2005) and may be associated with periodontal disease and its attenuation (Nakajima et al. 2005, Cardoso et al. 2008, Garlet et al. 2010). We did not find a change in Treg cells with *Aa* infection, which may be due to the length of the experiment, 4–6 weeks (Li et al. 2010). In mice, Tregs have been shown to be upregulated in approximately 9 weeks after *Aa* inoculation (Garlet et al. 2010). Haematologic examination of blood of *Aa*-infected rats revealed a significant increase ($p < 0.05$) in both monocyte percentage of WBCs and absolute numbers of monocytes. Although intriguing, the systemic changes noted do not necessarily reflect changes that are occurring in the periodontal tissue.

In conclusion, our studies demonstrate that outbred Wistar rats constitute a useful rodent model for studying *Aa*-induced periodontal disease, and our studies provide evidence that *Aa* infection induces a $CD8^+$ T cell response in draining lymph nodes and an increase in peripheral blood monocytes. Moreover, we found that *Aa* infection induced TNF-α expression in junctional epithelial cells and caused a loss of connective tissue attachment. Significantly, both were reversed by antibiotic treatment linking TNF-α expression in epithelial cells to this process providing insight into mechanisms of periodontal disease. Whether or not this particular animal model represents specific processes found in localized aggressive periodontitis remains to be proven.

Acknowledgements

We thank Harleen Gill for her assistance with the analysis of histologic specimens, Sunitha Batchu for helping with the preparation of the manuscript, the staff of the Comparative Medicine Facility of the New Jersey Medical School for their help with procedures involving the rats and Dr. Zoltan Spolarics and Stephanie Federici from the Department of Surgery at New Jersey Medical School, UMDNJ, for their assistance with the hematology assays.

References

Armitage, G. C. & Cullinan, M. P. (2010) Comparison of the clinical features of chronic and aggressive periodontitis. *Periodontology* **2000**, 12–27.

Artese, L., Simon, M. J., Piattelli, A., Ferrari, D. S., Cardoso, L. A., Faveri, M., Onuma, T., Piccirilli, M., Perrotti, V. & Shibli, J. A. (2011) Immunohistochemical analysis of inflammatory infiltrate in aggressive and chronic periodontitis: a comparative study. *Clinical Oral Investigation* **15**, 233–240.

Brown, L. J., Albandar, J. M., Brunelle, J. A. & Loe, H. (1996) Early-onset periodontitis: progression of attachment loss during 6 years. *Journal of Periodontology* **67**, 968–975.

Cardoso, C. R., Garlet, G. P., Moreira, A. P., Junior, W. M., Rossi, M. A. & Silva, J. S. (2008) Characterization of CD4 + CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. *Journal of Leukocyte Biology* **84**, 311–318.

Christersson, L. A., Slots, J., Rosling, B. G. & Genco, R. J. (1985) Microbiological and clinical effects of surgical treatment of localized juvenile periodontitis. *Journal of Clinical Periodontology* **12**, 465–476.

Cochran, D. L. (2008) Inflammation and bone loss in periodontal disease. *Journal of Periodontology* **79**(Suppl. 8), 1569–1576.

Darout, I. A., Albandar, J. M., Skaug, N. & Ali, R. W. (2002) Salivary microbiota levels in relation to periodontal status, experience of caries and miswak use in Sudanese adults. *Journal of Clinical Periodontology* **29**, 411–420.

Fine, D. H. & Furgang, D. (2002) Lactoferrin iron levels affect attachment of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells. *Journal of Periodontology* **73**, 616–623.

Fine, D. H., Goncharoff, P., Schreiner, H., Chang, K. M., Furgang, D. & Figurski, D. (2001) Colonization and persistence of rough and smooth colony variants of *Actinobacillus actinomycetemcomitans* in the mouths of rats. *Archives of Oral Biology* **46**, 1065–1078.

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., McKiernan, M. & Gunsolley, J. (2007) *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. *Journal of Clinical Microbiology* **45**, 3859–3869.

Fine, D. H., Velliyagounder, K., Furgang, D. & Kaplan, J. B. (2005) The *Actinobacillus actinomycetemcomitans* autotransporter adhesin Aae exhibits specificity for buccal epithelial cells from humans and old world primates. *Infection and Immunity* **73**, 1947–1953.

Garlet, G. P., Cardoso, C. R., Mariano, F. S., Claudino, M., de Assis, G. F., Campanelli, A. P., Avila-Campos, M. J. & Silva, J. S. (2010) Regulatory T cells attenuate experimental periodontitis progression in mice. *Journal of Clinical Periodontology* **37**, 591–600.

Goncharoff, P., Figurski, D. H., Stevens, R. H. & Fine, D. H. (1993) Identification of *Actinobacillus actinomycetemcomitans*: polymerase chain reaction amplification of lktA-specific sequences. *Oral Microbiology and Immunology* **8**, 105–110.

Graves, D. (2008) Cytokines that promote periodontal tissue destruction. *Journal of Periodontology* **79**, 1585–1591.

Graves, D. T., Fine, D., Teng, Y. T., Van Dyke, T. E. & Hajishengallis, G. (2008) The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. *Journal of Clinical Periodontology* **35**, 89–105.

Hamlet, S. M., Cullinan, M. P., Westerman, B., Lindeman, M., Bird, P. S., Palmer, J. & Seymour, G. J. (2001) Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. *Journal of Clinical Periodontology* **28**, 1163–1171.

Haubek, D., Ennibi, O. K., Poulsen, K., Vaeth, M., Poulsen, S. & Kilian, M. (2008) Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* in Morocco: a prospective longitudinal cohort study. *Lancet* **371**, 237–242.

Henderson, B., Ward, J. M. & Ready, D. (2010) *Aggregatibacter (Actinobacillus) actinomycetemcomitans:* a triple A* periodontopathogen? *Periodontology* **2000**(54), 78–105.

Li, Y., Messina, C., Bendaoud, M., Fine, D. H., Schreiner, H. & Tsiagbe, V. K. (2010) Adaptive immune response in osteoclastic bone resorption induced by orally administered *Aggregatibacter actinomycetemcomitans* in a rat model of periodontal disease. *Molecular Oral Microbiology* **25**, 275–292.

Lima, P. M., Souza, P. E., Costa, J. E., Gomez, R. S., Gollob, K. J. & Dutra, W. O. (2011) Aggressive and chronic periodontitis correlate with distinct cellular sources of key immunoregulatory cytokines. *Journal of Periodontology* **82**, 86–95.

Löe, H. & Brown, L. J. (1991) Early onset periodontitis in the United States of America. *Journal of Periodontology* **62**, 608–616.

Mandell, R. L. & Socransky, S. S. (1988) Microbiological and clinical effects of surgery plus doxycycline on juvenile periodontitis. *Journal of Periodontology* **59**, 373–379.

Moss-Salentijn, L. & Moss, M. L. (1977) Effects of occlusal attrition and continuous eruption on odontometry of rat molars. *American Journal of Physical Anthropology* **47**, 403–407.

Nakajima, T., Ueki-Maruyama, K., Oda, T., Ohsawa, Y., Ito, H., Seymour, G. J. & Yamazaki, K. (2005) Regulatory T-cells infiltrate periodontal disease tissues. *Journal of Dental Research* **84**, 639–643.

Paturel, L., Casalta, J. P., Habib, G., Nezri, M. & Raoult, D. (2004) *Actinobacillus actinomycetemcomitans* endocarditis. *Clinical Microbiology and Infection* **10**, 98–118.

Rudney, J. D., Chen, R. & Sedgewick, G. J. (2005) *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, and *Tannerella forsythensis* are components of a polymicrobial intracellular flora within human buccal cells. *Journal of Dental Research* **84**, 59–63.

Sakaguchi, S. (2005) Naturally arising Foxp3-expressing CD25 + CD4 + regulatory T cells in immunological tolerance to self and non-self. *Nature Immunonoly* **6**, 345–352.

Schenkein, H. A., Barbour, S. E. & Tew, J. G. (2007) Cytokines and inflammatory factors regulating immunoglobulin production in aggressive periodontitis. *Periodontology* **2000**(45), 113–27.

Schreiner, H. C., Sinatra, K., Kaplan, J. B., Furgang, D., Kachlany, S. C., Planet, P. J., Perez, B. A., Figurski, D. H. & Fine, D. H. (2003) Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proceedings of the National Academy of Sciences USA* **100**, 7295–7300.

Schreiner, H., Markowitz, K., Miryalkar, M., Moore, D., Diehl, S. & Fine, D. H. (2011) *Aggregatibacter actinomycetemcomitans*-induced bone loss and antibody response in three rat strains. *Journal of Periodontology* **82**, 142–150.

Sigusch, B. W., Wutzler, A., Nietzsch, T. & Glockmann, E. (2006) Evidence for a specific crevicular lymphocyte profile in aggressive periodontitis. *Journal of Periodontal Research* **41**, 391–396.

Slots, J., Reynolds, H. S. & Genco, R. J. (1980) *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infection and Immunity* **29**, 1013–1020.

Smith, P. C., Guerrero, J., Tobar, N., Cáceres, M., González, M. J. & Martínez, J. (2009) Tumor necrosis factor-α-stimulated membrane type 1-matrix metalloproteinase production is modulated by epidermal growth factor receptor signaling in human gingival fibroblasts. *Journal of Periodontal Research* **44**, 73–80.

Suda, R., Kurihara, C., Kurihara, M., Sato, T., Lai, C. H. & Hasegawa, K. (2003) Determination of eight selected periodontal pathogens in the subgingival plaque of maxillary first molars in Japanese school children aged 8-11 years. *Journal of Periodontal Research* **38**, 28–35.

Takamatsu, N., Yano, K., He, T., Umeda, M. & Ishikawa, I. (1999) Effect of initial periodontal therapy on the frequency of detecting *Bacteroides forsythus, Porphyromonas gingivalis,* and *Actinobacillus actinomycetemcomitans*. *Journal of Periodontology* **70**, 574–580.

Tonetti, M. S. & Mombelli, A. (1999) Early-onset periodontitis. *Annals of Periodontology* **4**, 39–53.

van Winkelhoff, A. J. & Boutaga, C. K. (2005) Transmission of periodontal pathogens and models of infection. *Journal of Clinical Periodontology* **32**(Suppl. 6), 16–27.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Histological sections of interproximal area in non-infected and infected animals.
Figure S2. Histological TRAP-stained sections of interproximal area in non-infected and infected animals.
Figure S3. Histological sections of interproximal area in non-infected and infected animals.
Figure S4. Histological TNF-α immunostained sections of interproximal area in non-infected and infected animals.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Address:
Dana T. Graves
University of Pennsylvania
School of Dental Medicine
240 South 40th Street
Philadelphia, PA 19104-6030
USA
E-mail: dtgraves@upenn.edu

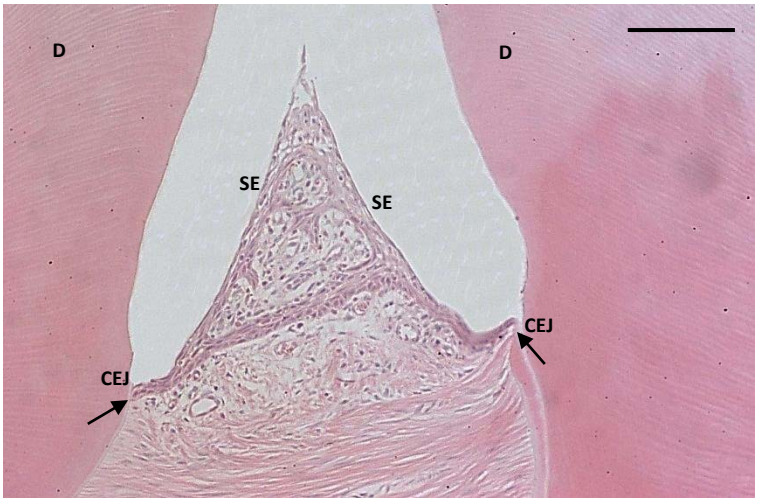
Clinical Relevance

Scientific rationale for the study: The local cellular events through which *Aa* infection leads to periodontitis have not been investigated using quantitative histologic analysis in the present animal model. As *Aa* is an important periodontal pathogen in localized juvenile periodontitis, the results give insight into the cellular events modulated by *Aa*.

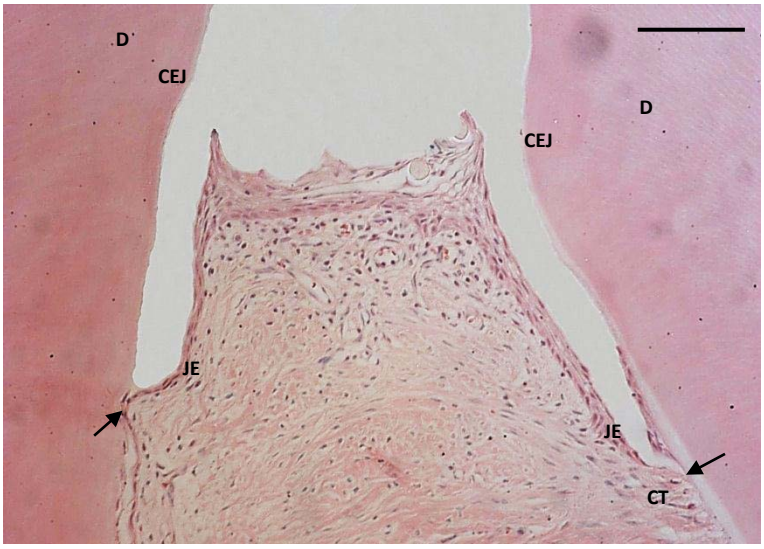
Principal findings: *Aa* infection altered the local response within the periodontium including the induction of cytokine expression, gingival inflammation and loss of attachment in conjunction with alveolar bone loss.

Practical implications: The *Aa* model is an important tool in understanding the pathogenesis of periodontitis in part because it is a natural host for *Aa*. The findings provide important characterization of the model that will give insight into human periodontitis.

Supplemental Figure 1



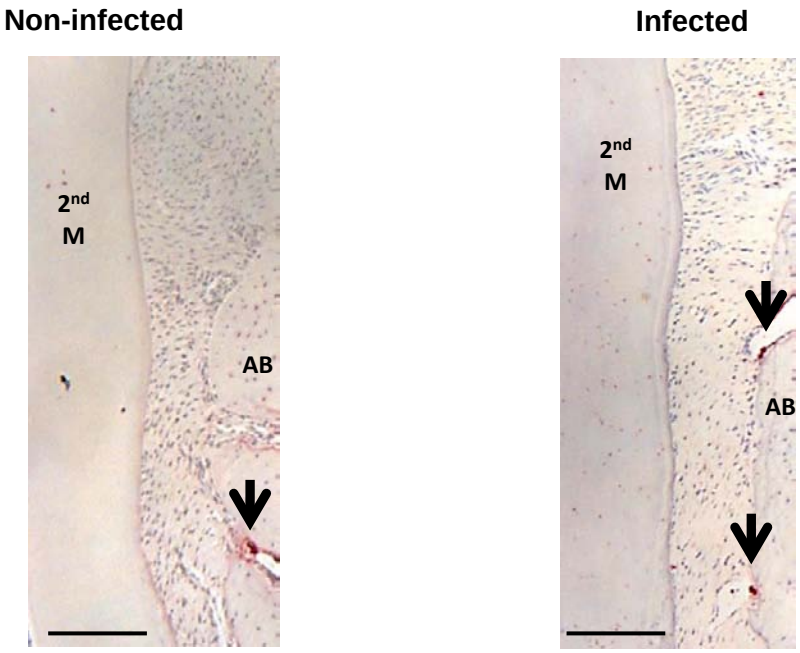
Non-infected



Infected

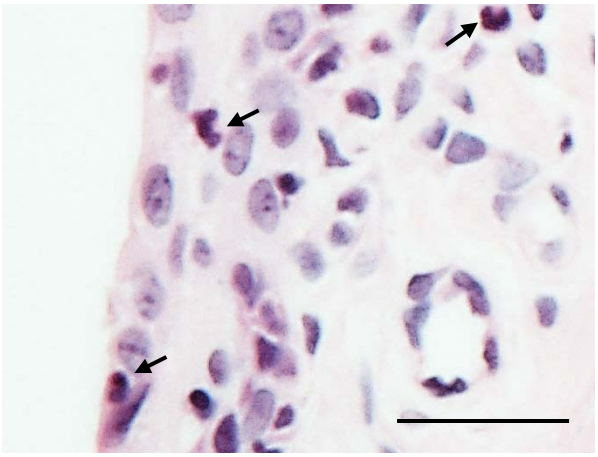
Histological sections of interproximal area in non-infected and infected animals. Infected animals showed greater attachment loss than non-infected ones. Arrows indicate the most coronal extent of connective tissue attachment to cementum. D- dentin; C- cementum; CEJ- cemento-enamel junction; SE- sulcular epithelium; CT- connective tissue; JE- junctional epithelium. Bars represent 0.05mm.

Supplemental Figure 2

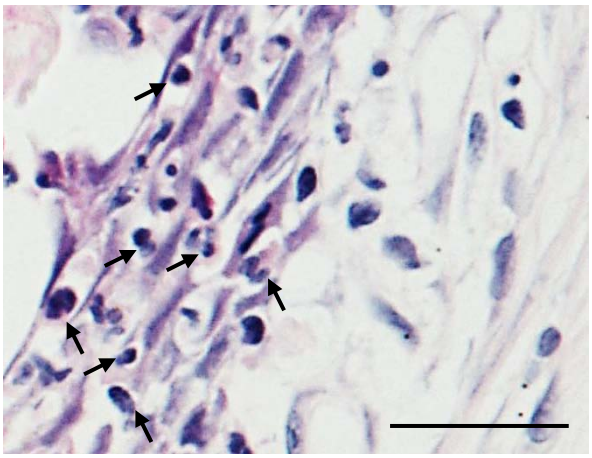


Histological TRAP stained sections of interproximal area in non-infected and infected animals. *Aa* infected animals had higher osteoclast numbers than non-infected ones. Arrow indicates osteoclast. 2nd M- second molar; AB- alveolar bone. Bar in lower left is 0.25mm.

Supplemental Figure 3



Epithelium non-infected

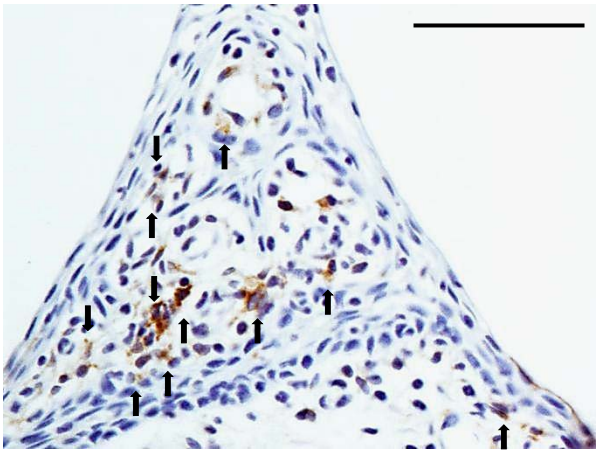


Epithelium infected

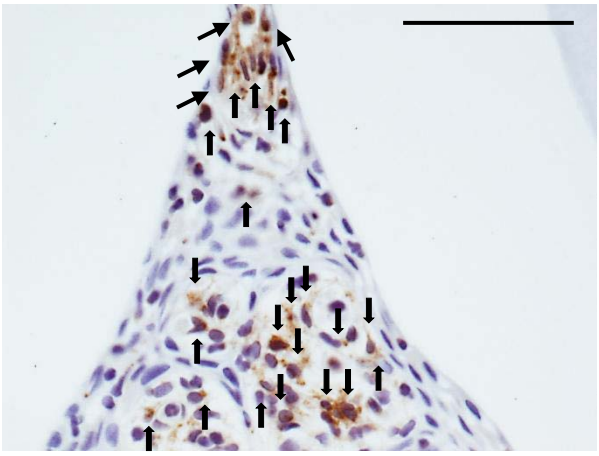
Histological sections of interproximal area in non-infected and infected animals. Infected animals showed greater infiltration of PMNs in the epithelium than non-infected ones. Arrows indicate polymorphonuclear cells in the interproximal gingiva. Bars represent 0.02mm.

Supplemental Figure 4

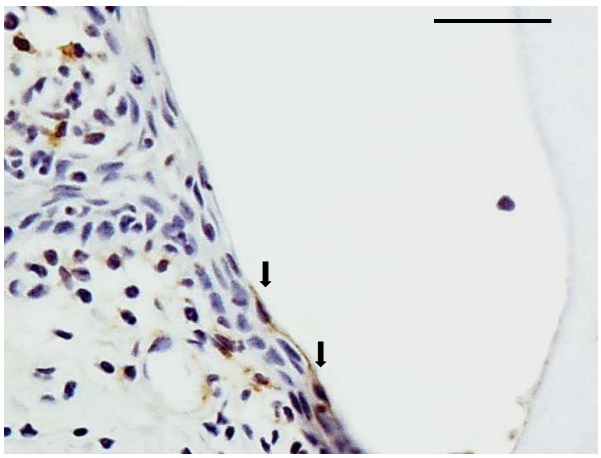
Gingival epithelium non-infected



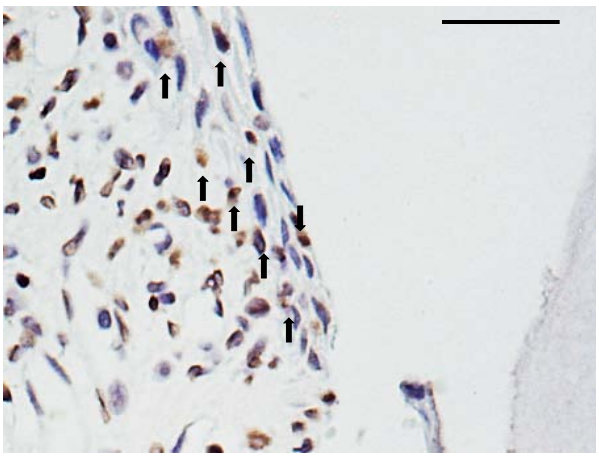
Gingival epithelium infected



Junctional Epithelium non-infected



Junctional Epithelium infected



Histological TNF-α immunostained sections of interproximal area in non-infected and infected animals. Infected animals showed greater number of cells expressing TNF-α than non-infected ones. Arrows indicate polymorphonuclear cells in the interproximal gingiva. Bars represent 0.04mm and 0.02mm in gingiva and junctional epithelium images, respectively.

Infection and Immunity

Aggregatibacter actinomycetemcomitans Infection Enhances Apoptosis *In Vivo* through a Caspase-3-Dependent Mechanism in Experimental Periodontitis

Jun Kang, Beatriz de Brito Bezerra, Sandra Pacios, Oelisoa Andriankaja, Yu Li, Vincent Tsiagbe, Helen Schreiner, Daniel H. Fine and Dana T. Graves

Infect. Immun. **2012, 80(6):2247. DOI: 10.1128/IAI.06371-11. Published Ahead of Print 26 March 2012.**

IAI Journals.ASM.org

Aggregatibacter actinomycetemcomitans Infection Enhances Apoptosis *In Vivo* through a Caspase-3-Dependent Mechanism in Experimental Periodontitis

Jun Kang,[a,b] Beatriz de Brito Bezerra,[c] Sandra Pacios,[b,e] Oelisoa Andriankaja,[b] Yu Li,[d] Vincent Tsiagbe,[d] Helen Schreiner,[d] Daniel H. Fine,[d] and Dana T. Graves[b]

Department of Periodontology, School and Hospital of Stomatology, Peking University, Beijing, China[a]; Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA[b]; Prosthodontics and Periodontics Department, Piracicaba Dental School, University of Campinas, Piracicaba, Brazil[c]; Department of Oral Biology, New Jersey Dental School, University of Medicine and Dentistry of New Jersey, Newark, New Jersey, USA[d]; and Department of Periodontology, School of Dental Medicine, Universitat Internacional de Catalunya, Sant Cugat del Vallès, Spain[e]

The purpose of this study was to test the hypothesis that diabetes aggravates periodontal destruction induced by *Aggregatibacter actinomycetemcomitans* infection. Thirty-eight diabetic and 33 normal rats were inoculated with *A. actinomycetemcomitans* and euthanized at baseline and at 4, 5, and 6 weeks after inoculation. Bone loss and the infiltration of polymorphonuclear leukocytes (PMNs) in gingival epithelium were measured in hematoxylin-eosin-stained sections. The induction of tumor necrosis factor alpha (TNF-α) was evaluated by immunohistochemistry and of apoptotic cells by a TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay. After *A. actinomycetemcomitans* infection, the bone loss in diabetic rats was 1.7-fold and the PMN infiltration 1.6-fold higher than in normoglycemic rats ($P < 0.05$). The induction of TNF-α was 1.5-fold higher and of apoptotic cells was up to 3-fold higher in diabetic versus normoglycemic rats ($P < 0.05$). Treatment with a caspase-3 inhibitor significantly blocked noninflammatory cell apoptosis induced by *A. actinomycetemcomitans* infection in gingival epithelium and connective tissue ($P < 0.05$). These results provide new insight into how diabetes aggravates *A. actinomycetemcomitans*-induced periodontal destruction in rats by significantly increasing the inflammatory response, leading to increased bone loss and enhancing apoptosis of gingival epithelial and connective tissue cells through a caspase-3-dependent mechanism. Antibiotics had a more pronounced effect on many of these parameters in diabetic than in normoglycemic rats, suggesting a deficiency in the capacity of diabetic animals to resist infection.

Periodontitis is one of the most prevalent infectious diseases worldwide. It is characterized by loss of supporting connective tissue and alveolar bone around the teeth (36). Although triggered by a bacterial infection, the destruction of periodontal tissue is caused by the inflammatory response to pathogenic bacteria. Immune mediators such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), IL-6, and RANKL have been found to be abundantly expressed in humans with periodontal disease, and increased levels have been shown in the crevicular fluid from patients with periodontitis (4, 13, 32). Animal studies have established cause-and-effect relationships between these cytokines and periodontal breakdown (13, 24).

There are several types of periodontal diseases ranging from chronic periodontitis that affects adults to a form of aggressive periodontitis that primarily affects adolescents, localized aggressive periodontitis (LAgP). LAgP is characterized by severe and rapid destruction of the supporting apparatus of the teeth, which may lead to tooth loss early in life (3). *Aggregatibacter actinomycetemcomitans* is commonly linked to LAgP (3, 16), and studies have shown that periodontal treatment leads to a reduction in its levels (5, 31). The presence of *A. actinomycetemcomitans* in periodontal pockets has also been considered indicative of future disease progression (9, 16). *A. actinomycetemcomitans* has virulence factors, such as leukotoxin and cytolethal distending toxin (CDT), that may contribute to its capacity to induce rapid tissue destruction by promoting apoptosis of a number of host cell types (20).

A rat model has been developed to study the pathogenic mechanisms of *A. actinomycetemcomitans*-induced periodontal tissue destruction (8, 28, 38, 39). This model is characterized by infecting the animals with a rough strain of *A. actinomycetemcomitans* that adheres to the oral epithelium and teeth (7). Although it may not mimic the specific form of periodontal disease found in localized aggressive periodontitis in humans, this model has provided insight into the colonization of the oral cavity by this bacterium and inflammation-induced periodontitis (28). However, relatively little is known about the local changes that are induced by this bacterium *in vivo*.

Periodontal disease is triggered by bacterial infection, but the local inflammatory response has been shown to mediate the actual destruction of periodontal tissue. This response can be modulated by systemic conditions such as diabetes. Diabetes has been identified as one of the important risk factors for periodontitis, increasing both its prevalence and severity (26, 27, 40). One mechanism through which diabetes increases periodontal tissue loss and other diabetic complications is by exacerbating the inflamma-

Received 29 December 2011 **Returned for modification** 16 February 2012
Accepted 20 March 2012
Published ahead of print 26 March 2012
Editor: B. A. McCormick
Address correspondence to Dana T. Graves, dtgraves@upenn.edu.
Supplemental material for this article may be found at http://iai.asm.org/.

doi:10.1128/IAI.06371-11

tory response to periodontal pathogens through increased oxidative stress, advanced glycation end products, and expression of cytokines such as TNF-α (12, 25, 33, 37).

Apoptosis is thought to contribute to periodontal disease progression. It has been suggested that apoptosis of epithelial cells may contribute to the loss of epithelial barrier function (6). Moreover, loss of gingival fibroblasts has been shown to be one of the largest cellular changes that occurs with periodontal disease progression and may be associated with a loss of connective tissue attachment (29, 43). Infection by *A. actinomycetemcomitans* has been shown to induce apoptosis *in vitro* (21–23). However, relatively little is known about how it induces apoptosis *in vivo* and how a systemic condition such as diabetes affects *A. actinomycetemcomitans*-induced apoptosis and periodontal tissue destruction. The experiments described here address these issues using diabetic and matched normoglycemic rats, which are natural hosts of *A. actinomycetemcomitans*. The results indicate that the effect of *A. actinomycetemcomitans* infection on bone loss, TNF-α expression, and apoptosis of epithelial cells and nonleukocytic gingival connective tissue cells is aggravated by diabetes. Moreover, we demonstrate that apoptosis is induced by a caspase-3-dependent mechanism.

MATERIALS AND METHODS

Animals. Goto-Kakizaki (GK) and normoglycemic control matched Wistar rats (5 to 10 weeks old) weighing 150 to 250 g were purchased from Charles River Laboratories (Wilmington, MA). The GK rat is a nonobese Wistar substrain that develops type 2 diabetes mellitus at age approximately 8 weeks. Rats were considered to be diabetic when glycated hemoglobin (HbAlc) levels exceeded 7.0%. During the experiments, the HbAlc level in GK rats was typically 7.0 to 10.5%. All normoglycemic rats had HbAlc levels that ranged from 4.3 to 4.8%. All animal procedures were approved by the Institutional Animal Care and Use Committee.

***A. actinomycetemcomitans* inoculation.** Both diabetic (GK) and normal (Wistar) rats were inoculated with *A. actinomycetemcomitans* as previously described (39). To depress the "natural" resident flora, rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days. During the last 2 days of antibiotic treatment, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex; Procter and Gamble, Cincinnati, OH). After a subsequent period of 3 days without antibiotic treatment, the rats were divided into six groups of approximately seven rats each. The adherent *A. actinomycetemcomitans* strain, Columbia University *A. actinomycetemcomitans* clinical isolate 1000 (CU1000NRif), was incubated in *A. actinomycetemcomitans* growth medium with 35 mg of rifampin (Sigma-Aldrich, St. Louis, MO)/ml for 2 days. Adherent cells in culture dishes were scraped into a solution of phosphate-buffered saline (PBS) plus 3% sucrose, and minor adjustment was made by the addition of buffer to obtain 10^8 cells/ml (optical density at 560 nm = 0.80). After fasting for 3 h, rats received 10^8 *A. actinomycetemcomitans* cells in 1 g of powdered food supplemented with 3% sucrose. This protocol was followed for 4 days and was repeated the following week for a total of eight *A. actinomycetemcomitans* inoculations in food (39). During the first 4 days of the feeding, the rats also received 10^8 *A. actinomycetemcomitans* in PBS by oral gavage. After 1 h, the inoculated food was removed and replaced with regular powdered food. Rats were euthanized 4, 5, and 6 weeks after the inoculation period was completed. Baseline animals did not receive *A. actinomycetemcomitans* in their food and were not inoculated with *A. actinomycetemcomitans* but did receive powered food supplemented with 3% sucrose under the same conditions as the experimental rats.

Treatment with antibiotics and caspase-3 inhibitor. At 4 weeks after *A. actinomycetemcomitans* inoculation, two groups of rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days with the intention to reduce the infection. Concomitantly, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex).

Caspase-3 inhibitor (Z-DEVD-FMK; SM Biochemicals, Anaheim, CA) was administered by intraperitoneal injection (1.5 mg/kg). Control animals were injected with the same volume of vehicle (2% dimethyl sulfoxide; MP Biomedicals, Solon, OH). The caspase-3 inhibitor was administrated 1 week before euthanasia, and it was injected daily until the animals were euthanized.

Sampling of total anaerobic bacteria (CFU) and detection of *A. actinomycetemcomitans* by PCR. Two microbial samples were collected: one after the inoculation of *A. actinomycetemcomitans* and the other at the time of euthanasia. The rats were anesthetized, and their oral microflora was sampled using a cotton tip swab for soft tissue sampling and a toothpick (Johnson & Johnson, Piscataway, NJ) for hard tissue sampling. Both samples were combined in tubes containing 1 ml of PBS. Serial 10-fold dilutions were made and plated on Trypticase soy agar with 5% sheep blood (BD Biosciences, San Jose, CA) for total anaerobe counts. Trypticase soy agar plates were incubated in an anaerobic atmosphere at 37°C for 7 days to obtain total bacterial counts. To detect whether *A. actinomycetemcomitans* was present in the samples, DNA was prepared directly from the collected oral samples with a DNA extraction kit (Qiagen, Valencia, CA) and subjected to PCR analysis using forward and reverse primers (5′-GGAATTCCTAGGTATTGCGAAACAATTTGATC-3′ and 5′-GGAATTCCTGAAATTAAGCTGGTAATC-3′, respectively), which amplified a 262-bp PCR product from the *A. actinomycetemcomitans* leukotoxin gene as previously described (10).

Level of antibody to *A. actinomycetemcomitans*. IgG antibody reactive with *A. actinomycetemcomitans* was assessed by enzyme-linked immunosorbent assay (ELISA). Blood was collected by cardiac puncture and serum was obtained and stored at −20°C. An *A. actinomycetemcomitans* lysate was prepared and used to coat the wells of microtiter dishes (Nunc-ImmunoPlate with a Maxi Sorp surface; Thermo Fisher Scientific, Rochester, NY). A standard curve was generated using purified rat IgG (Sigma-Aldrich) in carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich). Rat serum diluted 1/5 and 1/10 in blocking buffer was added to the wells coated with *A. actinomycetemcomitans* pellet lysate. The serum dilutions were added in duplicate wells, washed, incubated with rabbit anti-rat IgG-Fc conjugated to alkaline phosphatase (Bethyl Laboratories, Montgomery, TX), and quantified with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). Absorbance was read on a microplate reader at 405 nm.

Histomorphometric analysis of hematoxylin-eosin-stained sections. Right maxillas were fixed in 4% paraformaldehyde at 4°C for 48 h and decalcified in 10% EDTA (pH 7.0) for 12 weeks. Paraffin-embedded sagittal sections were prepared at a thickness of 5 μm. The mid-interproximal region between first and second molars was examined in each specimen and was established by being sectioned to a level where the root canal systems in adjacent teeth were visible. Two randomly chosen sections of each interproximal area were examined at ×200 magnification. All data were analyzed by a blinded examiner who did not know the group to which an animal belonged. Bone loss was measured as the distance between the cemento-enamel junction (CEJ) and the highest peak of the interproximal bone. The number of polymorphonuclear (PMNs) leukocytes was counted in the gingival epithelium at ×600 magnification. The identification of these cells was confirmed by an experienced examiner.

Histomorphometric analysis of TNF-α immunohistochemistry-stained sections. To evaluate the number of cells expressing TNF-α, sections were stained by immunohistochemistry with an antibody against TNF-α (IHC World, Woodstock, MD). The number of positive cells was evaluated 1 mm down from the level of bone crest apically in an area of periodontal ligament between the first and second molars. Cell counts were obtained by one examiner and confirmed by a second independent examiner with similar results. The numbers of positive cells in epithelium and gingival connective tissue were scored based on the following scale: 0, no positive cells; 1, three to four positive cells per field with weak immu-

nostaining; 2, four to ten positive cells per field with strong immunostaining; and 3, more than ten positive cells per field with strong immunostaining. Sections were examined at ×600 magnification.

Detection of apoptotic cells. Apoptotic cells were detected by an *in situ* TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay (DeadEnd fluorometric TUNEL system kit; Promega, Madison, WI) according to the manufacturer's instructions. This kit detects double-strand breaks in genomic DNA and identifies most stages of apoptosis. The fluorescein-12-dUTP-labeled DNA was then visualized directly by fluorescence microscopy. Additional counts were made to specifically avoid counting apoptotic leukocytes. This was accomplished by using the TUNEL assay, followed by immunofluorescence with an anti-CD18 antibody (Novus Biological, Littleton, CO). The number of nonleukocytic apoptotic cells ($TUNEL^+$ $CD18^-$) was counted at ×200 magnification with an immunofluorescence microscope using NIS Elements software (Nikon, Melville, NY) in epithelium or connective tissue above the alveolar bone crest. Cells counts were obtained by one examiner and confirmed by a second independent examiner with similar results.

Systemic leukocyte analysis. Rat lymph leukocytes were isolated and analyzed as previously described (28). Single-cell suspensions were obtained from the submandibular and cervical lymph nodes. Lymphocyte populations were isolated by Ficoll-Hypaque density gradient centrifugation. Flow cytometry was conducted using anti-CD32 (clone D34-485) for blocking FcγII receptors, phycoerythrin (PE)-conjugated anti-CD4 (clone OX-38), fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone G4.18; BD Biosciences); FITC-labeled anti-FoxP3 (clone FJK-16S), and PE-conjugated anti-CD25 (clone OX39) from eBioscience (San Diego, CA), and anti-IA (clone 14-4-4S) from the American Type Culture Collection (Manassas, VA). Blood was analyzed by HemaTrue hematology analyzer (Heska, Loveland, CO). The total numbers of white blood cells, the numbers of lymphocytes, monocytes, and granulocytes, and the percentages of lymphocytes, monocytes, and granulocytes were analyzed.

Statistical analysis. Differences between two groups such as diabetic and normal rats were determined by using the Student *t* test and between time points within a group by one-way analysis of variance except for the evaluation of TNF-α. Differences in TNF-α values were determined by nonparametric analysis with Mann-Whitney U test. Significance levels were set at 5%.

RESULTS

Induction of periodontal disease. At baseline, the antibody titer level in diabetic rats was low and increased after *A. actinomycetemcomitans* infection so that at 6 weeks it was 32-fold higher than at baseline ($P < 0.01$) (Fig. 1A). Moreover, for all *A. actinomycetemcomitans* infected diabetic rats, the antibody titers were 18-fold higher than for the noninfected animals ($P < 0.01$) (Fig. 1B). Also, after infection diabetic the animals had antibody titers level that were 2.3-fold higher than normoglycemic infected animals ($P < 0.05$) (Fig. 1B). Diabetic rats also showed a significant decrease in antibody titer level after antibiotic treatment ($P < 0.05$) (Fig. 1C).

The impact of *A. actinomycetemcomitans* infection on total anaerobic bacteria levels in noninfected normoglycemic and diabetic rats, as well as infected diabetic rats, was also examined. Despite a trend toward increased levels of anaerobic bacteria in infected diabetic rats compared to normoglycemic rats, the differences were not significant (see Table S1 in the supplemental material). Similarly the percentage of rats exposed to *A. actinomycetemcomitans* that had detectable infection was not higher between the normoglycemic and diabetic groups (see Table S2 in the supplemental material).

A number of parameters was evaluated to examine the impact of *A. actinomycetemcomitans* infection on the systemic leukocyte

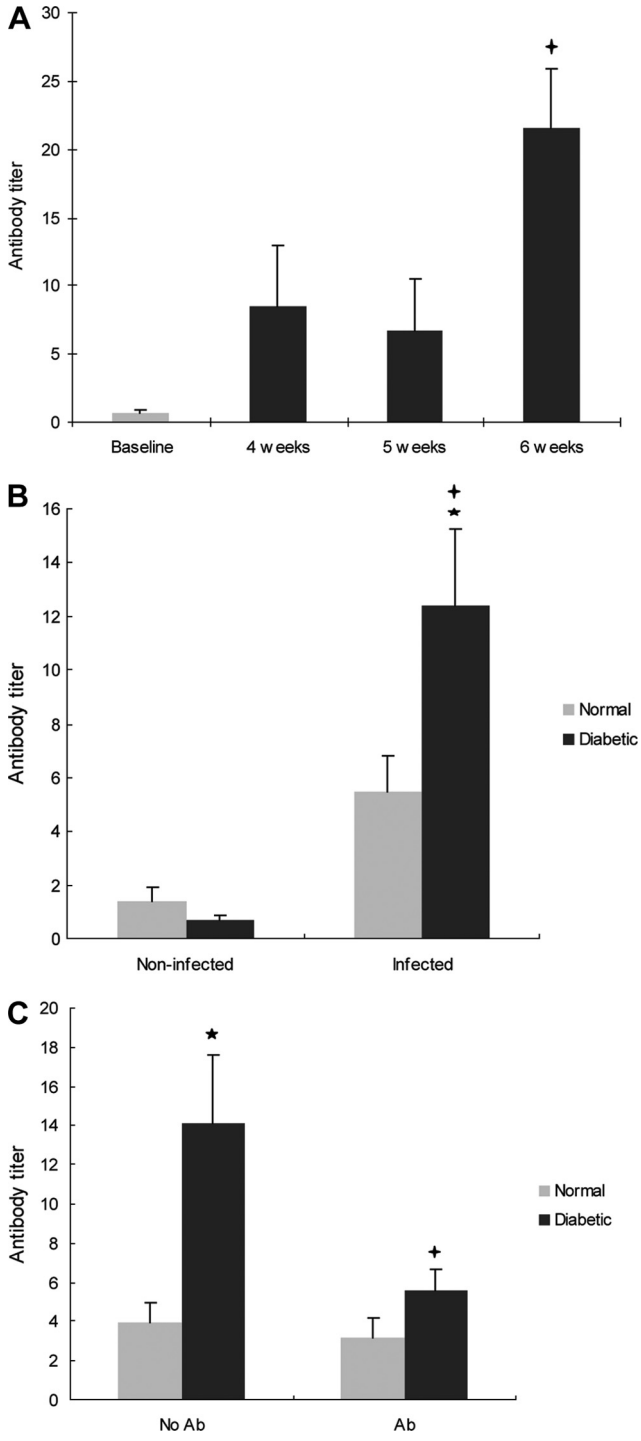


FIG 1 Diabetes increases the antibody titer to *A. actinomycetemcomitans* in infected rats. Diabetic and normal rats were infected orally with *A. actinomycetemcomitans*, and antibody (IgG) reactive with *A. actinomycetemcomitans* was assessed by ELISA. At 4 weeks postinfection, one group of rats was treated with antibiotics or equivalent vehicle alone. Rats were euthanized at baseline and at 4, 5, and 6 weeks after *A. actinomycetemcomitans* inoculation was completed. (A) Antibody titer levels in diabetic rats over time. (B) Antibody titer in noninfected (baseline) and infected (4 to 6 weeks) normoglycemic and diabetic rats. (C) Effect of antibiotic treatment on antibody titer in normoglycemic and diabetic rats. Each value in panels A, B, and C is the mean of five to seven rats ± the standard error of the mean (SEM). *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic rats in different groups ($P < 0.05$).

TABLE 1 Lymphocyte populations of noninfected and *A. actinomycetemcomitans*-infected rats[a]

	Mean ± SEM			
	Normal rats		Diabetic rats	
Type	Noninfected	Infected	Noninfected	Infected
MHC II	55.9 ± 11.7	44.1 ± 14.8	38.3 ± 6.8*	37.0 ± 12.0
$CD4^+$	31.5.1 ± 11.4	32.4 ± 13.0	42.1 ± 9.3	42.7 ± 13.9
$CD8^+$	24.6 ± 6.9	35.2 ± 10.7	29.6 ± 9.4	31.4 ± 8.9
$CD25^+$	5.2 ± 0.9	6.7 ± 2.8	5.4 ± 0.8	7.9 ± 2.4
$FoxP3^+$	4.3 ± 0.8	5.3 ± 3.1	7.0 ± 3.4	7.2 ± 2.4

[a] Lymphocyte populations from draining cervical and submandibular lymph nodes were analyzed as described in Materials and Methods. The rats described in Fig. 1 were examined for lymphocyte populations according to infection status. Each value is the mean of five to seven rats. *, $P < 0.05$, compared to normal rats.

populations in diabetic animals. After *A. actinomycetemcomitans* infection there was no change in *A. actinomycetemcomitans*-infected rats compared to uninfected rats of major histocompatibility complex (MHC) class II-positive cells, T cells, B cells, or Treg cells for either normoglycemic or diabetic rats (Tables 1 and 2). However, there was a slight reduction in the percentage of lymphocytes in the peripheral circulation of infected diabetic rats compared to infected normoglycemic rats and a 1.5-fold increase in the percentage of granulocytes in infected diabetic rats compared to infected normoglycemic rats ($P < 0.05$) (Table 2).

Bone loss was induced in the diabetic rats, as evidenced by an increase in the distance from the CEJ to the alveolar bone crest after 5 weeks ($P < 0.05$) (Fig. 2A and see Fig. S1 in the supplemental material). In *A. actinomycetemcomitans*-infected diabetic animals there was a 1.8-fold increase in bone loss compared to noninfected diabetic rats at baseline. The bone loss was 1.7-fold higher in the infected diabetic rats compared to infected normoglycemic rats ($P < 0.05$) (Fig. 2B). Antibiotic treatment significantly decreased the bone loss in the diabetic rats ($P < 0.05$) (Fig. 2C).

PMN infiltration. The formation of a PMN infiltrate in gingival epithelium was assessed (see Fig. S2 in the supplemental material). PMNs increased 7-fold ($P < 0.05$) 4 weeks after *A. actinomycetemcomitans* inoculation in diabetic rats (Fig. 3A). Both

TABLE 2 Leukocytic cells of noninfected and *A. actinomycetemcomitans*-infected rats[a]

	Mean ± SEM			
	Normal		Diabetic	
Parameter	Noninfected	Infected	Noninfected	Infected
No. (10^3 UI/ml)				
WBC	7.9 ± 2.9	7.8 ± 4.7	6.9 ± 3.2	8.0 ± 4.0
Lymphocytes	6.2 ± 2.5	5.8 ± 3.3	4.4 ± 1.0	5.2 ± 2.8
Monocytes	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.2
Granulocytes	1.3 ± 0.5	1.7 ± 1.5	2.2 ± 2.0	2.3 ± 1.4
Amt (%)				
Lymphocytes	77.0 ± 5.5	76.0 ± 10.5	69.0 ± 13.8	65.6 ± 13.5*
Monocytes	4.8 ± 1.0	3.5 ± 1.2	4.8 ± 1.4	4.5 ± 1.8
Granulocytes	18.1 ± 4.6	20.6 ± 9.9	26.2 ± 12.5	30.0 ± 13.4*

[a] Leukocytic cells from peripheral blood were analyzed as described in Materials and Methods. The rats described in Fig. 1 were examined for leukocytic cells according to infection status. Each value is the mean of five to seven rats. *, $P < 0.05$, compared to normal rats. WBC, white blood cells.

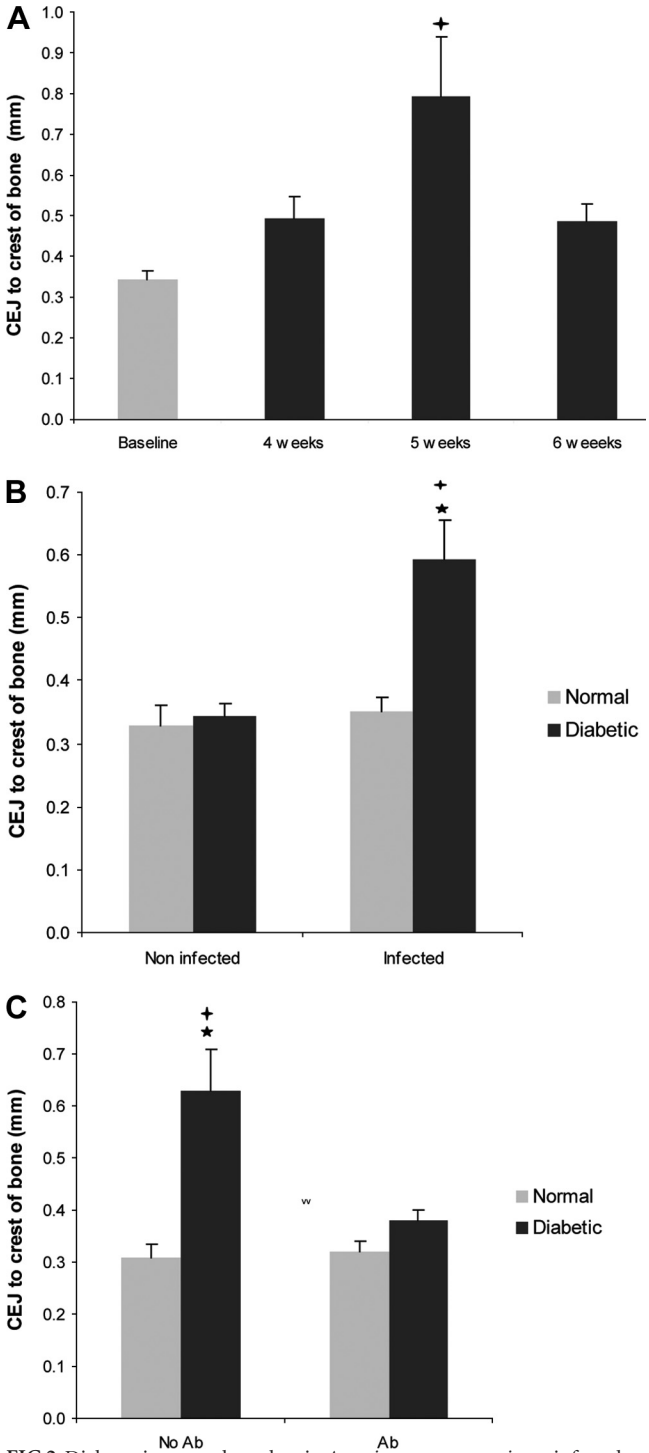


FIG 2 Diabetes increases bone loss in *A. actinomycetemcomitans*-infected rats. The distance between the CEJ and the alveolar bone crest was measured. (A) CEJ-to-bone distance in diabetic rats. (B) CEJ-to-bone distance in noninfected (baseline) and infected (4 to 6 weeks) normoglycemic and diabetic rats. (C) Effect of antibiotic treatment on CEJ-to-bone distance in normoglycemic and diabetic rats. Each value is the mean of five to seven rats ± the SEM. *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic rats in different groups ($P < 0.05$).

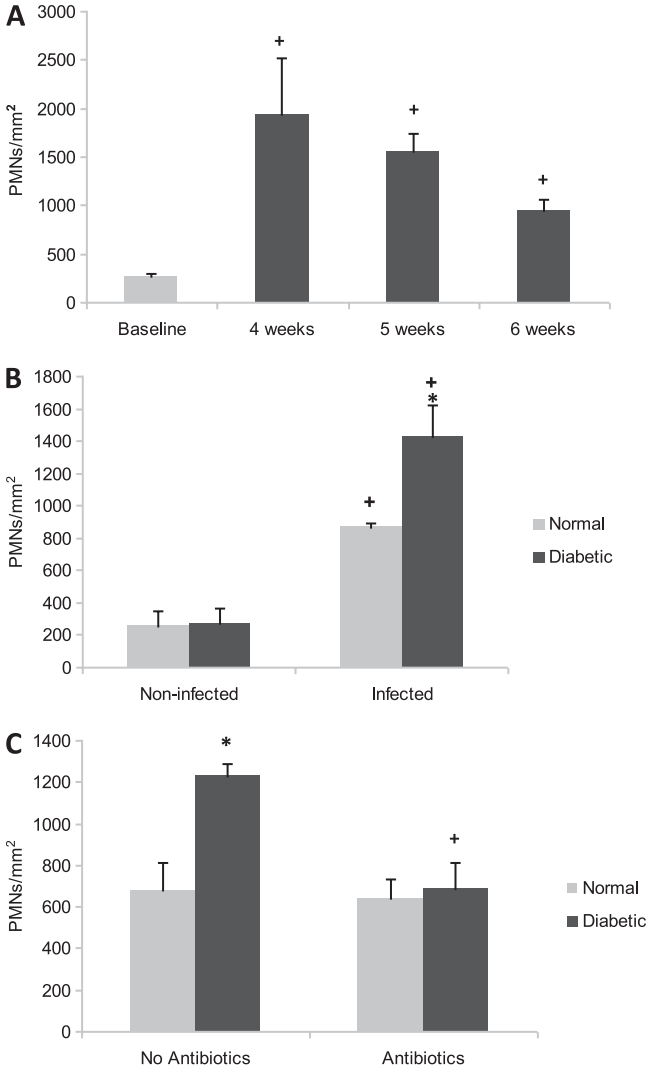


FIG 3 Diabetes increases the number of PMNs of *A. actinomycetemcomitans*-infected rats. The number of PMNs infiltrating the gingival epithelium was measured. (A) PMN infiltration in diabetic rats over time. (B) PMNs in noninfected (baseline) and infected (4 to 6 weeks) normoglycemic and diabetic rats. (C) Effect of antibiotic treatment on PMN infiltration in normoglycemic and diabetic rats. Each value in panels A, B, and C is the mean of five to seven rats ± the SEM. *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic rats in different groups ($P < 0.05$).

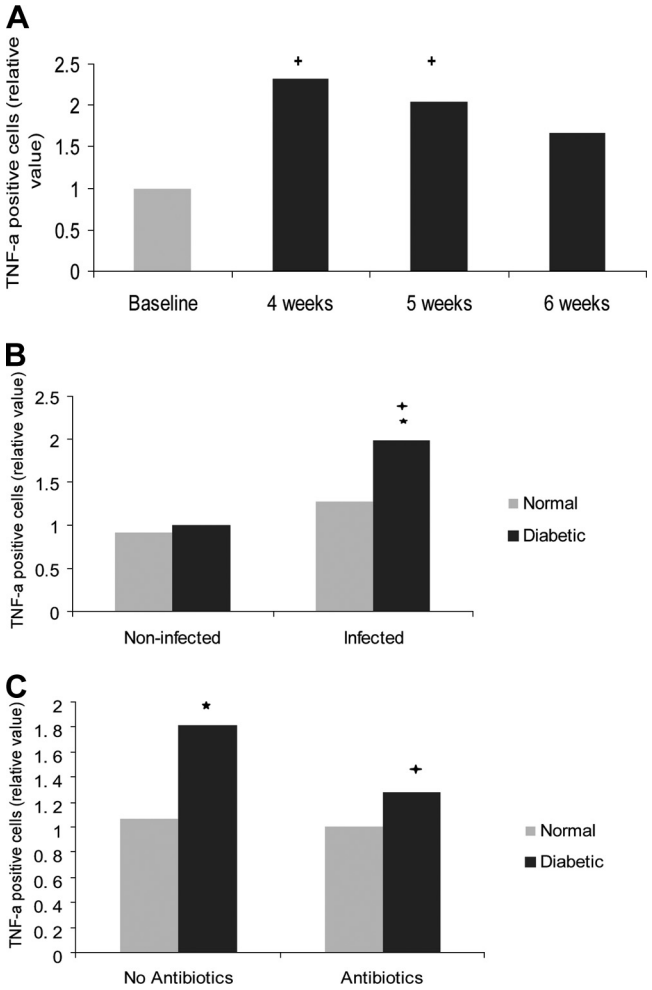


FIG 4 Diabetes increases the TNF-α expression in the gingival epithelia of *A. actinomycetemcomitans*-infected rats. TNF-α-positive cells were detected by immunohistochemistry in histologic specimens using a specific antibody. The rats described in Fig. 1 were examined for TNF-α expression using the following scale that took both the number of immunopositive cells and the intensity of the immunostaining into account: 0, no positive cells; 1, three to four positive cells per field with weak immunostaining; 2, four to ten positive cells per field with strong immunostaining; and 3, more than ten positive cells per field with strong immunostaining. (A) TNF-α expression in the gingival epithelia of diabetic rats. (B) TNF-α expression in noninfected (baseline) and infected (4 to 6 weeks) gingival epithelia in normoglycemic and diabetic rats. (C) Effect of antibiotic treatment in normoglycemic and diabetic rats. Each value represents the mean of 5 to 7 rats ± the SEM. *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic or normal rats in different groups ($P < 0.05$).

normal and diabetic noninfected rats had similar levels of PMNs. After *A. actinomycetemcomitans* infection PMN numbers increased 3.4-fold in normal rats, while it increased 5.3-fold in diabetic rats ($P < 0.05$) (Fig. 3B). The greater increase in PMNs in infected diabetic rats, compared to infected normal rats, is consistent with the significant increase ($P < 0.05$) in blood granulocytes in infected diabetic rats. Antibiotic treatment significantly reduced the PMN infiltration in the diabetic rats ($P < 0.05$) (Fig. 3C).

TNF-α. TNF-α was measured in the gingival epithelium and connective tissue. In the epithelium, the TNF-α values of diabetic rats were significantly higher in animals exposed to *A. actinomycetemcomitans* inoculation compared to noninfected diabetic rats ($P < 0.05$) and significantly higher than in infected normoglycemic rats ($P < 0.05$) (Fig. 4B and see Fig. S3 in the supplemental material). Antibiotic treatment resulted in a significant decrease in TNF-α expression in the epithelium of diabetic rats ($P < 0.05$) but had no effect in the normoglycemic group (Fig. 4C).

TNF-α was also measured in the gingival connective tissue. It significantly increased in diabetic rats 5 weeks after *A. actinomycetemcomitans* infection ($P < 0.05$) (Fig. 5A) and was substantially higher than values found in infected normoglycemic rats ($P < 0.05$) (Fig. 5B). When rats were treated with antibiotic, there were no differences in TNF-α levels in the connective tissues of diabetic and normal rats ($P > 0.05$) (Fig. 5C).

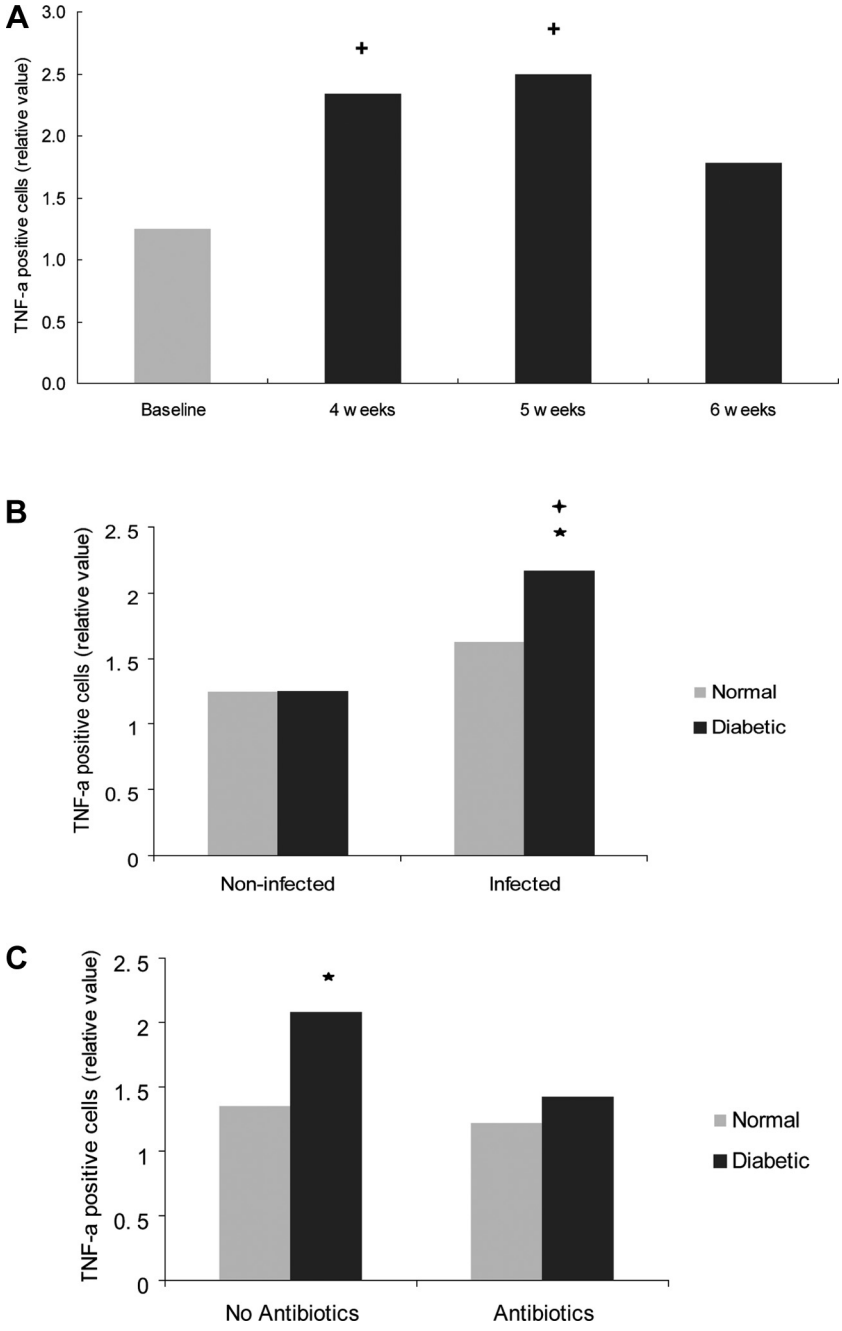


FIG 5 TNF-α expression is increased in gingival connective tissues of diabetic rats following *A. actinomycetemcomitans* infection. (A) TNF-α expression in gingival connective tissues of diabetic rats. (B) TNF-α expression in noninfected (baseline) and infected (4–6 weeks) gingival connective tissues in normoglycemic and diabetic rats. (C) Effect of antibiotic treatment in gingival connective tissues of normoglycemic and diabetic rats. Each value represents the mean of five to seven rats ± the SEM. *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic or normal rats in different groups ($P < 0.05$).

Induction of apoptosis. Because apoptosis is thought to play an important role in periodontal disease progression, we sought to determine whether diabetic animals had significantly higher levels of apoptosis in the gingival epithelium (see Fig. S4 in the supplemental material) and whether the increase was mediated by caspase-3 in both. Prior to *A. actinomycetemcomitans* infection, the level of apoptosis was low in both diabetic and normoglycemic groups. The onset of *A. actinomycetemcomitans* infection significantly increased the level of apoptosis 2- to 3-fold in the normoglycemic rats and 12-fold in the diabetic rats, with the difference between the two groups being significant ($P < 0.05$) (Fig. 6A). The results were similar when presented as the percentage of gingival epithelial cells that were apoptotic or the number of apoptotic epithelial cells/μm^2 (Fig. 6B). The principal leukocytic cell type infiltrating *A. actinomycetemcomitans*-infected gingiva was the granulocyte. Apoptosis was evaluated as the percentage of

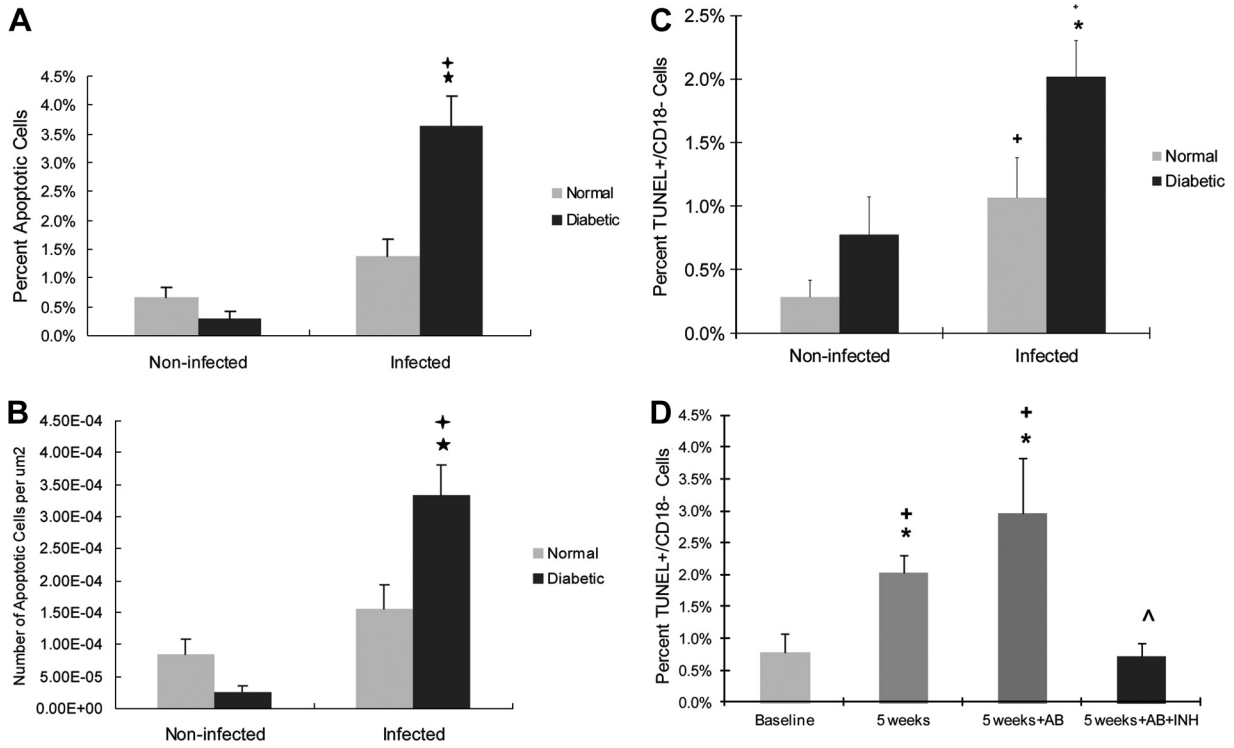


FIG 6 Diabetes increases the apoptosis of epithelial cells of *A. actinomycetemcomitans*-infected rats in a caspase-3-dependent manner. Apoptotic cells were detected in gingival epithelium by TUNEL staining in the epithelia in the rats described in Fig. 1. In some groups, rats were treated with antibiotic or antibiotic plus capase-3 inhibitor starting at week 4. (A) Percentage of apoptotic gingival epithelial cells per total number of gingival epithelial cells; (B) total number of apoptotic gingival epithelial cells per area. (C and D) Nonleukocytic cells were identified as CD18 negative, and apoptotic cells were identified as TUNEL positive. (C) TUNEL$^+$ CD18$^-$ cells per total number of CD18$^-$ cells. (D) Rats at week 4 were treated with antibiotic or antibiotic plus caspase-3 inhibitor and euthanized a week later. The TUNEL$^+$ CD18$^-$ cells per total number of CD18$^-$ cells were counted. Each value is the mean of five to seven rats ± the SEM. *, significant difference between diabetic and normal rats ($P < 0.05$); +, significant difference between diabetic or normal rats in different groups ($P < 0.05$); ^, significant difference between antibiotic and antibiotic plus caspase-3 inhibitor.

nonleukocytic TUNEL$^+$ CD18$^-$ cells in the gingival epithelium. The number of TUNEL$^+$ CD18$^-$ epithelial cells was significantly increased in both the normal and diabetic groups after *A. actinomycetemcomitans* infection ($P < 0.05$) (Fig. 6C). The percentage of apoptotic cells in diabetic animals was 2-fold greater than in normoglycemic animals ($P < 0.05$) (Fig. 6C). To assess the impact of inhibiting caspase-3/7, the specific caspase inhibitor DEVD was administered daily, starting at week 4, and the number of TUNEL$^+$ CD18$^-$ cells was counted 1 week later. *A. actinomycetemcomitans* infection at this time point increased the apoptosis of epithelial cells by 2.6-fold compared to the baseline, but antibiotic treatment had no significant effect in reducing these levels ($P > 0.05$). Treatment with caspase inhibitor plus antibiotics reduced the number of apoptotic epithelial cells by reversing the impact of *A. actinomycetemcomitans* infection to baseline levels ($P < 0.05$) (Fig. 6D).

Apoptosis was also examined in gingival connective tissue. Both normal and diabetic rats showed an almost 3-fold increase in apoptotic cells after infection when examined as the percentage of positive cells or as the number of apoptotic cells per area ($P < 0.05$) (Fig. 7A and B). The total numbers of apoptotic cells in the gingival connective tissue of the diabetic group were >2-fold higher than for the normoglycemic rats ($P < 0.05$). The percentage of TUNEL$^+$ CD18$^-$ cells in the gingival connective tissue was measured. After *A. actinomycetemcomitans* infection, the values increased 3.9-fold ($P < 0.05$) in diabetic animals but not in normal animals (Fig. 7C). At 5 weeks after *A. actinomycetemcomitans* infection, the percentage of TUNEL$^+$ CD18$^-$ cells significantly increased in the diabetic group ($P < 0.05$) (Fig. 7D). Antibiotic treatment alone had no effect, but antibiotic treatment combined with caspase-3/7 inhibitor significantly blocked the increase in nongranulocytic cell apoptosis in the connective tissue ($P < 0.05$) (Fig. 7D).

DISCUSSION

The results presented here demonstrate that *A. actinomycetemcomitans* infection significantly enhances PMN infiltration and TNF-α expression in both normal and diabetic rats. Moreover, each of these parameters was significantly greater in the diabetic animals, a finding which agrees with the increased bone loss observed in the diabetic group here as well as in other studies (17, 29, 30). Thus, diabetic rats exhibited greater inflammatory responses compared to the normoglycemic group in response to similar *A. actinomycetemcomitans* inocula.

Diabetes generally enhances inflammation by altering myeloid and lymphoid functions (12, 34). We found here that the local periodontal inflammatory response in diabetic animals was greater, as evidenced by an enhanced expression of TNF-α and a larger PMN infiltrate, and this finding is consistent with findings in other models (15, 33). These local findings were in agreement

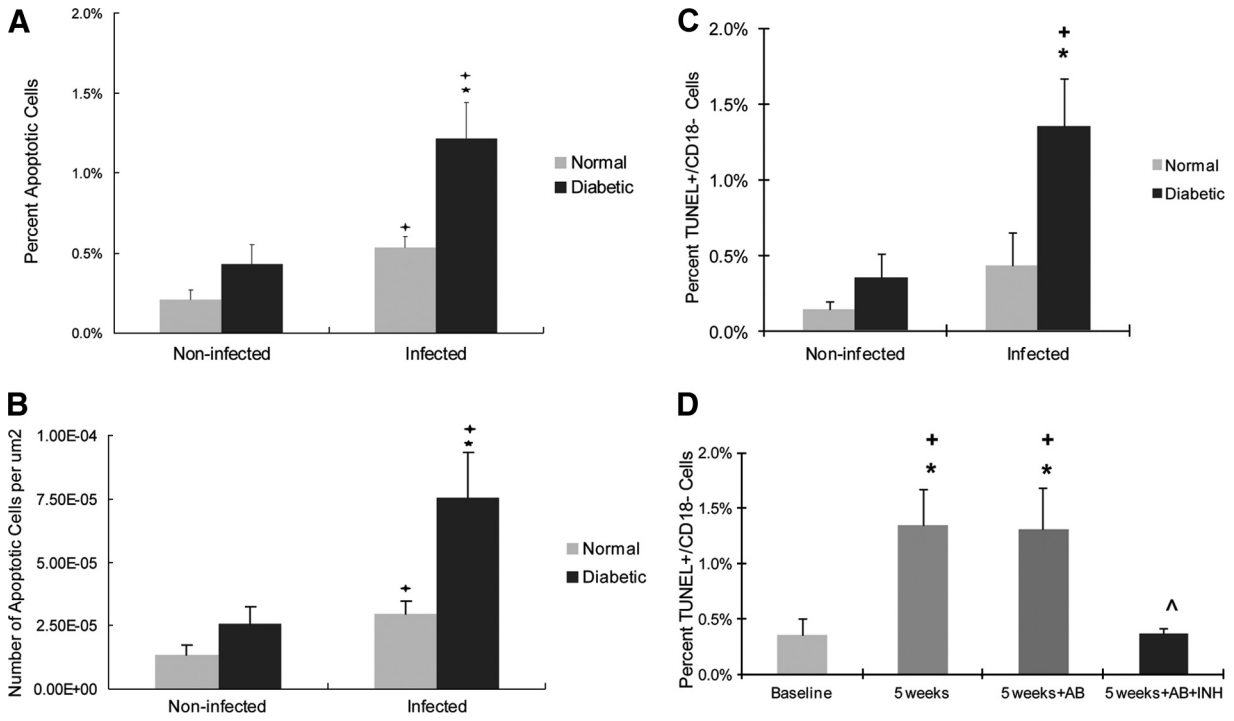


FIG 7 Diabetes increases apoptosis of cells in gingival connective tissue of *A. actinomycetemcomitans*-infected rats in a caspase-3-dependent manner. (A) Total apoptotic gingival connective tissue cells per total number of gingival connective tissue cells. (B) Total apoptotic gingival connective tissue cells per epithelial area. (C and D) Nonleukocytic cells were identified as CD18 negative, and apoptotic cells were identified as TUNEL positive. (C) $TUNEL^+$ $CD18^-$ cells per total number of $CD18^-$ cells in connective tissue. (D) Rats at week 4 were treated with antibiotic or antibiotic and caspase-3 inhibitor and euthanized a week later. The $TUNEL^+$ $CD18^-$ cells per total number of $CD18^-$ cells were counted. Each value is the mean of five to seven rats ± the SEM. *, significant difference between diabetics and normal rats ($P < 0.05$); +, significant difference between diabetics or normal rats in different groups ($P < 0.05$); ^, significantly different between antibiotic and antibiotic plus caspase-3 inhibitor.

with the significant increase in the percentage of whole-blood granulocytes in diabetic rats postinfection. Elevated levels of antibody against *A. actinomycetemcomitans* were also found in diabetic rats postinfection compared to normal rats. The number of lymphocytes collected from whole blood, however, did not exhibit the same trend, showing a significant decrease compared to normoglycemic rats after *A. actinomycetemcomitans* infection. It is conceivable that this decrease in lymphocyte population could be a result of CDT-induced apoptosis. Alternatively, the decrease in lymphocyte population after *A. actinomycetemcomitans* infection may be due to a proportional increase in granulocytes.

A. actinomycetemcomitans infection has been shown to increase apoptosis *in vitro* but has not yet been tested *in vivo* in a periodontal model (20). We demonstrate here that inoculating animals with *A. actinomycetemcomitans* significantly stimulated apoptosis in both the gingival epithelium and connective tissue of rats, especially in diabetic animals. Other studies have also shown that apoptosis is significantly increased in diabetes when periodontal disease is induced in an animal model (14, 29). There are several mechanisms through which *A. actinomycetemcomitans* infection could enhance apoptosis in the rat. Our study indicates that the high rate of apoptosis in diabetic rats due to *A. actinomycetemcomitans* infection is largely blocked by a caspase-3/7 inhibitor. It is possible that *A. actinomycetemcomitans* through its cytolethal distending toxin (CDT) could stimulate apoptosis. CDT has been shown to induce apoptosis in epithelial cells, fibroblasts, and endothelial cells (19, 35). It has recently been shown that CDT induces apoptosis through a caspase-3-dependent pathway in immortalized gingival epithelial cells (1). However, the other apoptosis-inducing factor produced by *A. actinomycetemcomitans*, leukotoxin A, appears not to stimulate apoptosis in rat cells (20). Alternatively, *A. actinomycetemcomitans* could induce apoptosis through indirect mechanisms. Interestingly, diabetic rats had significantly higher TNF-α levels and more apoptotic cells compared to normal rats after *A. actinomycetemcomitans* infection. TNF-α has been shown to mediate both *P. gingivalis*- and lipopolysaccharide-induced apoptosis *in vivo* (2, 11). Thus, excessive production of TNF-α is another potential pathway through which diabetes could enhance the apoptosis of epithelial and connective tissue cells, thereby affecting the response to bacterial infection, and may occur simultaneously with CDT-induced apoptosis.

The impact of antibiotic treatment postinfection was also evaluated. Antibiotics have long been used as an adjunct therapy in the treatment of localized aggressive periodontitis (18, 41, 42). We also examined the impact of antibiotic treatment on *A. actinomycetemcomitans* antibody titer, alveolar bone resorption, PMN infiltration, TNF-α levels, and apoptosis in *A. actinomycetemcomitans*-infected periodontium. For *A. actinomycetemcomitans* antibody titer, PMN infiltration, TNF-α levels, and apoptosis, the diabetic rats showed a significant reduction with antibiotic treatment, whereas these parameters were not reduced by antibiotic treatment in normoglycemic rats. These results suggest that there are antibacterial deficits in diabetic animals that contribute to greater induction of proinflammatory events stimulated by peri-

odontal pathogens at the local level that can be reversed by antibiotic treatment.

In summary, the impact of diabetes on the periodontium was investigated in a relatively new model of periodontitis, oral inoculation of *A. actinomycetemcomitans* in the rat, which has the advantage that the rat is a natural host of *A. actinomycetemcomitans*. In this model, diabetes affected *A. actinomycetemcomitans*-induced periodontal destruction by significantly increasing the inflammatory response, leading to increased bone loss and apoptosis of gingival epithelial and connective tissue cells. The excessive production of TNF-α and the impact of CDT could be potential mechanisms through which apoptosis was induced at higher levels in diabetic animals. Antibiotics were able to reverse many parameters of the local host response in diabetic animals compared to normoglycemic animals, suggesting that a component to the enhanced inflammatory response is due to a deficit in the capacity of diabetic animals to resist infection. This information provides valuable insight as to how diabetes may alter host-bacterium interactions in a way that promotes periodontal breakdown.

ACKNOWLEDGMENTS

This study was supported by grants DE018307 and DE017732 from the NIDCR. Beatriz de Brito Bezerra was the recipient of a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brazil).

We thank Sunitha Batchu for helping with the preparation of the manuscript, the staff of the Comparative Medicine Facility of the New Jersey Medical School for their help with procedures involving the animal, and Zoltan Spolarics and Stephanie Federici of Department Surgery at New Jersey Medical School (UMDNJ) for their assistance with the hematology assays.

REFERENCES

1. **Alaoui-El-Azher M, et al.** 2010. Role of the ATM-checkpoint kinase 2 pathway in CDT-mediated apoptosis of gingival epithelial cells. PLoS One **5**:e11714.
2. **Alikhani M, et al.** 2003. Lipopolysaccharides indirectly stimulate apoptosis and global induction of apoptotic genes in fibroblasts. J. Biol. Chem. **278**:52901–52908.
3. **Armitage GC, Cullinan MP.** 2010. Comparison of the clinical features of chronic and aggressive periodontitis. Periodontol. 2000 **53**:12–27.
4. **Boch JA, Wara-aswapati N, Auron PE.** 2001. Interleukin 1 signal transduction: current concepts and relevance to periodontitis. J. Dent. Res. **80**:400–407.
5. **Christersson LA.** 1993. *Actinobacillus actinomycetemcomitans* and localized juvenile periodontitis: clinical, microbiologic, and histologic studies. Swed. Dent. J. Suppl. **90**:1–46.
6. **Dickinson BC, et al.** 2011. Interaction of oral bacteria with gingival epithelial cell multilayers. Mol. Oral Microbiol. **26**:210–220.
7. **Fine DH, Furgang D.** 2002. Lactoferrin iron levels affect attachment of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells. J. Periodontol. **73**:616–623.
8. **Fine DH, et al.** 2001. Colonization and persistence of rough and smooth colony variants of *Actinobacillus actinomycetemcomitans* in the mouths of rats. Arch. Oral Biol. **46**:1065–1078.
9. **Fine DH, et al.** 2007. *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. J. Clin. Microbiol. **45**:3859–3869.
10. **Goncharoff P, Figurski DH, Stevens RH, Fine DH.** 1993. Identification of *Actinobacillus actinomycetemcomitans*: polymerase chain reaction amplification of lktA-specific sequences. Oral Microbiol. Immunol. **8**:105–110.
11. **Graves D, et al.** 2001. Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to *P. gingivalis* infection. J. Dent. Res. **80**:1875–1879.
12. **Graves DT, Kayal RA.** 2008. Diabetic complications and dysregulated innate immunity. Front. Biosci. **13**:1227–1239.
13. **Graves DT, Li J, Cochran DL.** 2011. Inflammation and uncoupling as mechanisms of periodontal bone loss. J. Dent. Res. **90**:143–153.
14. **Graves DT, Liu R, Oates TW.** 2007. Diabetes-enhanced inflammation and apoptosis: impact on periodontal pathosis. Periodontol. 2000 **45**:128–137.
15. **Graves DT, et al.** 2005. Inflammation is more persistent in type 1 diabetic mice. J. Dent. Res. **84**:324–328.
16. **Haubek D, et al.** 2008. Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* in Morocco: a prospective longitudinal cohort study. Lancet **371**:237–242.
17. **He H, et al.** 2004. Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss. Endocrinology **145**:447–452.
18. **Heller D, et al.** 2011. Impact of systemic antimicrobials combined with anti-infective mechanical debridement on the microbiota of generalized aggressive periodontitis: a 6-month RCT. J. Clin. Periodontol. **38**:355–364.
19. **Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE.** 2011. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology **157**:1851–1875.
20. **Kachlany SC.** 2010. *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. J. Dent. Res. **89**:561–570.
21. **Kasai H, Yamamoto K, Koseki T, Yokota M, Nishihara T.** 2004. Involvement of caspase activation through release of cytochrome *c* from mitochondria in apoptotic cell death of macrophages infected with *Actinobacillus actinomycetemcomitans*. FEMS Microbiol. Lett. **233**:29–35.
22. **Kato S, et al.** 1995. Evidence for apoptosis of murine macrophages by *Actinobacillus actinomycetemcomitans* infection. Infect. Immun. **63**:3914–3919.
23. **Kato S, Sugimura N, Nakashima K, Nishihara T, Kowashi Y.** 2005. *Actinobacillus actinomycetemcomitans* induces apoptosis in human monocytic THP-1 cells. J. Med. Microbiol. **54**:293–298.
24. **Kirkwood KL, Cirelli JA, Rogers JE, Giannobile WV.** 2007. Novel host response therapeutic approaches to treat periodontal diseases. Periodontol. 2000 **43**:294–315.
25. **Lalla E, et al.** 2000. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. J. Clin. Invest. **105**:1117–1124.
26. **Lalla E, Papapanou PN.** 2011. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat. Rev. Endocrinol. **7**:738–748.
27. **Lamster IB, Lalla E, Borgnakke WS, Taylor GW.** 2008. The relationship between oral health and diabetes mellitus. J. Am. Dent. Assoc. **139**(Suppl):19S–24S.
28. **Li Y, et al.** 2010. Adaptive immune response in osteoclastic bone resorption induced by orally administered *Aggregatibacter actinomycetemcomitans* in a rat model of periodontal disease. Mol. Oral Microbiol. **25**:275–292.
29. **Liu R, et al.** 2006. Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. J. Dent. Res. **85**:510–514.
30. **Mahamed DA, et al.** 2005. G(−) anaerobes-reactive $CD4^+$ T cells trigger RANKL-mediated enhanced alveolar bone loss in diabetic NOD mice. Diabetes **54**:1477–1486.
31. **Mandell RL, Socransky SS.** 1988. Microbiological and clinical effects of surgery plus doxycycline on juvenile periodontitis. J. Periodontol. **59**:373–379.
32. **Mogi M, et al.** 1999. Interleukin 1 beta, interleukin 6, beta 2-microglobulin, and transforming growth factor-alpha in gingival crevicular fluid from human periodontal disease. Arch. Oral Biol. **44**:535–539.
33. **Naguib G, Al-Mashat H, Desta T, Graves D.** 2004. Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. J. Invest. Dermatol. **123**:87–92.
34. **Nikolajczyk BS, Jagannathan-Bogdan M, Shin H, Gyurko R.** 2011. State of the union between metabolism and the immune system in type 2 diabetes. Genes Immun. **12**:239–250.
35. **Ohara M, Miyauchi M, Tsuruda K, Takata T, Sugai M.** 2011. Topical application of *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin induces cell cycle arrest in the rat gingival epithelium in vivo. J. Periodontal Res. **46**:389–395.
36. **Pihlstrom BL, Michalowicz BS, Johnson NW.** 2005. Periodontal diseases. Lancet **366**:1809–1820.
37. **Salvi G, et al.** 1997. Monocytic TNFalpha secretion patterns in IDDM patients with periodontal diseases. J. Clin. Periodontol. **24**:8–16.
38. **Schreiner H, et al.** 2011. Aggregatibacter *actinomycetemcomitans-*

induced bone loss and antibody response in three rat strains. J. Periodontol. **82**:142–150.
39. **Schreiner HC, et al.** 2003. Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. Proc. Natl. Acad. Sci. U. S. A. **100**:7295–7300.
40. **Taylor G, et al.** 1998. Non-insulin-dependent diabetes mellitus and alveolar bone loss progression over 2 years. J. Periodontol. **69**:76–83.
41. **Xajigeorgiou C, Sakellari D, Slini T, Baka A, Konstantinidis A.** 2006. Clinical and microbiological effects of different antimicrobials on generalized aggressive periodontitis. J. Clin. Periodontol. **33**:254–264.
42. **Yek EC, et al.** 2010. Efficacy of amoxicillin and metronidazole combination for the management of generalized aggressive periodontitis. J. Periodontol. **81**:964–974.
43. **Zappa U, Reinking-Zappa M, Graf H, Chase D.** 1992. Cell populations associated with active probing attachment loss. J. Periodontol. **63**:748–752.

Supplemental Table 1. Anaerobe counts of *Aa* non-infected and infected rats (CFU)

	Non-infected	Infected
Normal	2825±1753	3928±2654
Diabetic	3325±2960	11374±17800

The anaerobes in oral microflora were counted as described in MATERIALS and METHODS. The diabetic and normal rats were infected orally with *Aa* since 4 weeks. Rats were sacrificed before (baseline) and after infection (4, 5 and 6 weeks). The anaerobes were counted according to status of infection. Each value is the mean of 5 to 7 rats ± SE.

Supplemental Table 2. *Aa* positive percentage of rats before and after *Aa* infection

	Non-infected			Infected		
	***Aa*+**	***Aa*-**	***Aa*+ percent**	***Aa*+**	***Aa*-**	***Aa*+ percent**
Normal	**0**	**7**	**0%**	**17**	**10**	**62.96%**
Diabetic	**0**	**7**	**0%**	**17**	**6**	**73.91%**

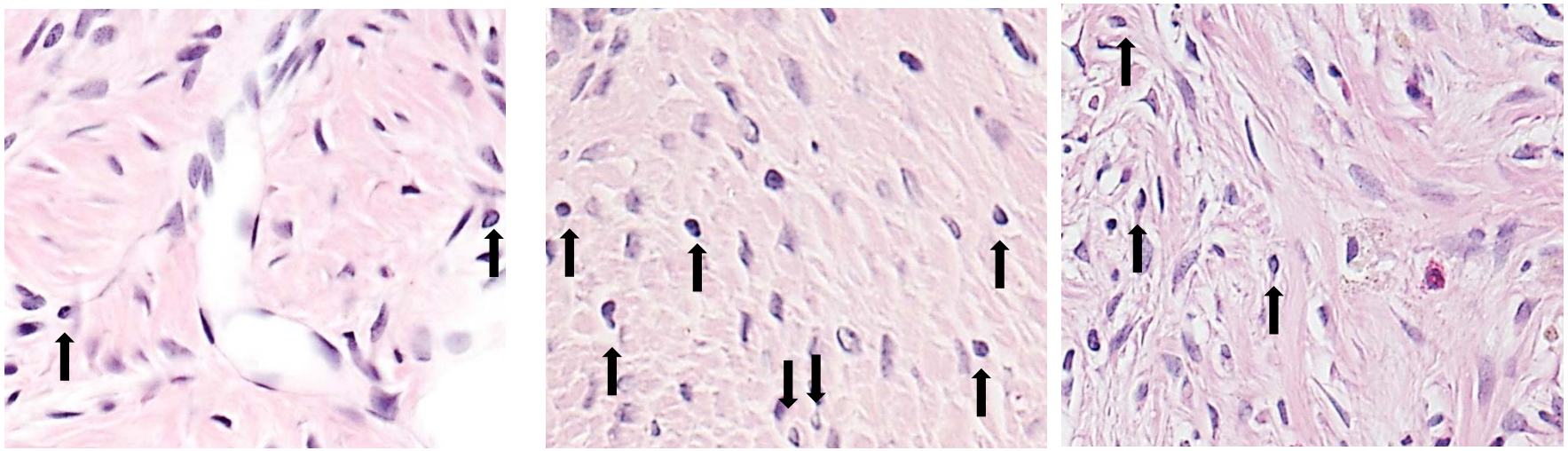
Rats were determined to be either *Aa* positive or *Aa* negative as described in Materials and Methods).

Supplemental Figure 1

H &E stained sagittal section taken from GK diabetic rats at baseline, 5 weeks following *Aa* infection and *Aa* infection with antibiotic treatment for the last week. B= bone

Supplemental Figure 2

Baseline Infected Antibiotic

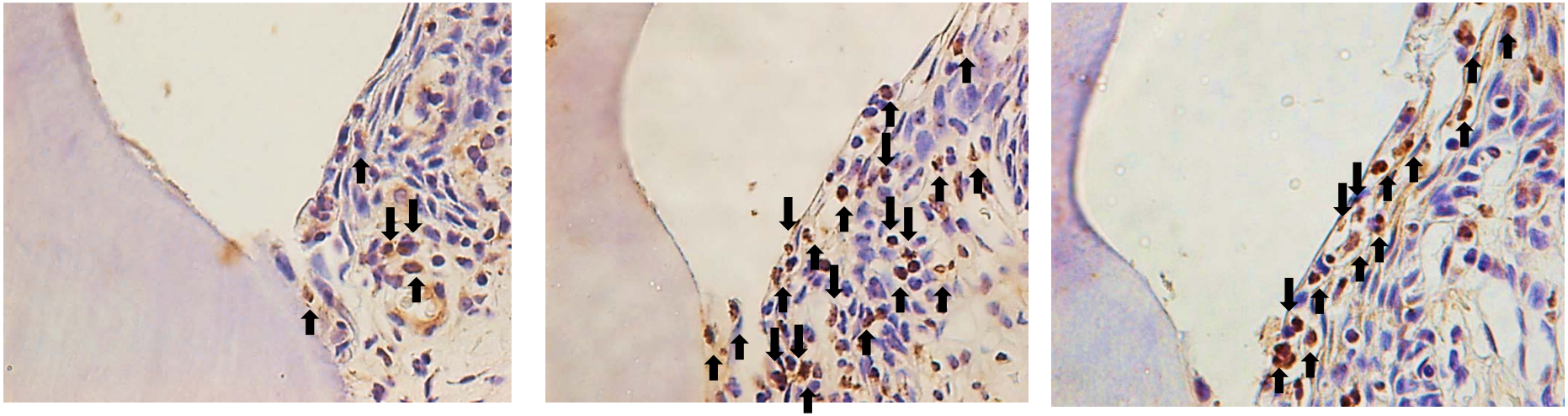


H &E stained sagittal section taken from sub-epithelial gingival connective tissue of GK diabetic rats at baseline, 5 weeks following *Aa* infection and *Aa* infection with antibiotic treatment for the last week. Arrows point to polymorphonuclear leukocytes.

Supplemental Figure 3

TNF-α

Baseline | Infected | Antibiotic



TNF expression in the epithelium of GK diabetic rats was assessed by immunohistochemisty with an anti-TNF-a specific antibody at baseline, 5 weeks following *Aa* infection and *Aa* infection with antibiotic treatment for the last week. No immunopositive cells were detected with matched control antibody (data not shown). Arrows point to TNF-α immunopositive cells (but not all positive cells).

Supplemental Figure 4

Apoptotic cells in the gingival epithelium of diabetic rats were detected by the TUNEL assay in histologic sections taken at baseline, 5 weeks following *Aa* infection and *Aa* infection with antibiotic treatment for the last week. Sections were counterstained with DAPI to show nuclei. Arrows point to TUNEL positive cells.

Diabetes aggravates periodontitis by limiting repair through enhanced inflammation

Sandra Pacios,*,‡,1 Jun Kang,*,§,1 Johnah Galicia,* Kenneth Gluck,‖ Hemal Patel,* Amy Ovaydi-Mandel,‖ Sophia Petrov,‖ Faizan Alawi,† and Dana T. Graves*,2

*Department of Periodontics and †Department of Pathology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA; ‡Department of Periodontics School of Dental Medicine, Universitat Internacional de Catalunya, Sant Cugat del Vallès, Spain; §Department of Periodontology, School and Hospital of Stomatology, Peking University, Beijing, China; and ‖Department of Periodontics New Jersey Dental School, University of Medicine and Dentistry of New Jersey (UMDNJ), Newark, New Jersey, USA

ABSTRACT **Periodontitis is the most common lytic bone disease and one of the first clinical manifestations of diabetes. Diabetes increases the risk of periodontitis. The aim of the present study was to examine mechanisms by which diabetes aggravates periodontitis. Ligature-induced periodontitis was examined in Goto-Kakizaki rats with type 2 diabetes. A tumor necrosis factor (TNF)-specific-inhibitor, pegsunercept, was applied to diabetic rats after the onset of periodontal disease. Interferon-γ (IFN-γ), TNF-α, interleukin-1 β (IL-1β), fibroblast growth factor-2 (FGF-2), transforming growth factor beta-1 (TGFβ-1), bone morphogenetic protein-2 (BMP-2), and BMP-6 were measured by real-time RT-PCR, and histological sections were examined for leukocyte infiltration and several parameters related to bone resorption and formation. Inflammation was prolonged in diabetic rats and was reversed by the TNF inhibitor, which reduced cytokine mRNA levels, leukocyte infiltration, and osteoclasts. In contrast, new bone and osteoid formation and osteoblast numbers were increased significantly *vs.* untreated diabetic animals. TNF inhibition in diabetic animals also reduced apoptosis, increased proliferation of bone-lining cells, and increased mRNA levels of FGF-2, TGFβ-1, BMP-2, and BMP-6. Thus, diabetes prolongs inflammation and osteoclastogenesis in periodontitis and through TNF limits the normal reparative process by negatively modulating factors that regulate bone.—Pacios, S., Kang, J., Galicia, J., Gluck, K., Patel, H., Ovaydi-Mandel, A., Petrov, S., Alawi, F., Graves, D. T. Diabetes aggravates periodontitis by limiting repair through enhanced inflammation. *FASEB J.* 26, 1423–1430 (2012). www.fasebj.org**

Key Words: bone formation · cytokine · growth factor · inhibitor

PERIODONTITIS IS THE MOST COMMON lytic disease of bone and a frequent complication of diabetes (1, 2). Individuals with diabetes have increased risk and severity of periodontal disease (1, 2). Moreover, it has been reported that periodontitis is one of the first clinical manifestations of diabetes (2). Periodontitis involves the loss of supporting structure for the tooth consisting of connective tissue attachment and bone. The control of this disease is important in maintaining the integrity and function of the oral cavity.

Periodontal disease is initiated by bacteria that induce an inflammatory response that causes tissue destruction. More than 700 bacterial species can be found in the oral cavity, yet only a small percentage of these are thought to initiate periodontal disease (3). The process of periodontitis involves colonization of the tooth surface, penetration of the connective tissue by bacteria or their products, and stimulation of inflammation that induces periodontal destruction and might also limit the repair process (4). The inflammatory response, rather than the direct pathological effects of the bacteria, is thought to cause the tissue destruction of periodontal disease, as shown by inhibiting prostaglandins and cytokines or through the action of anti-inflammatory lipid mediators (5–8).

Periodontal disease in humans and experimental animal models is linked to both the innate and adaptive immune response. Several studies have reported that individuals with periodontitis exhibit increased levels of interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6) in gingiva and crevicular fluid, which is a gingival exudate (9). Both genetic deletion and specific inhibition of these cytokines have been found to reduce periodontal disease progression (6, 9–11). Similarly, mediators of the adaptive immune response are elevated in individuals with periodontal

Abbreviations: BMP, bone morphogenetic protein; DM, diabetes mellitus; FGF-2, fibroblast growth factor-2; GK, Goto-Kakizaki; H&E, hematoxylin and eosin; LPS, lipopolysaccharide; PCNA, proliferating cell nuclear antigen; PDL, periodontal ligament; PMN, polymorphonuclear; RAGE, receptor for advanced glycation end-product; RANKL, receptor activator of nuclear factor κ-B ligand; TGFβ-1, transforming growth factor β-1; TRAP, tartrate-resistant acid phosphatase.

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Periodontics, University of Pennsylvania, 240 S. 40th St., Levy 122, Philadelphia, PA 19104, USA. E-mail: dtgraves@dental.upenn.edu

doi: 10.1096/fj.11-196279

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

disease, and inhibition or genetic deletion of mediators, such as receptor activator of nuclear factor κ-B ligand (RANKL) and interferon-γ (IFN-γ), result in reduced periodontal disease progression (10, 12, 13).

Several studies demonstrate that the prevalence and severity of periodontal disease are greater in patients with diabetes mellitus (DM) and in animal models of diabetes (1, 2). Diabetes may affect periodontal disease through a number of different avenues, including an impaired antibacterial defense. However, studies investigating whether diabetes causes a change in oral flora have had inconsistent results. An alternative explanation is that diabetes alters the inflammatory response to periodontal pathogens. This finding is supported by studies examining the response to a well-defined inoculum of periodopathic bacteria (14). Under normal circumstances, inflammation normally resolves through an active process regulated by cellular signals (15). However, resolution of inflammation is impaired in the diabetic animals. A greater inflammatory response explains the loss of periodontal tissues, since inflammatory mediators stimulate the production of lytic enzymes that break down connective tissue and formation of osteoclasts that resorb bone, both of which are hallmarks of periodontal disease.

One of the inflammatory mediators that has been implicated in the tendency of diabetics to have greater periodontal disease is TNF-α. Cell cultures studies have shown that lipopolysaccharide (LPS)-stimulated monocytes from individuals with type 1 diabetes produce a greater amount of TNF-α than monocytes from nondiabetic individuals (16). Moreover, TNF-α has been shown to mediate the destructive events induced by exposure to periodontal pathogens (17, 18). In addition, higher levels of TNF-α have been noted in a type 1 diabetes mouse model of periodontal disease that was linked to greater receptor for advanced glycation end-product (RAGE) signaling (19). However, it is not known whether elevated TNF-α contributes to the diabetic complication of enhanced periodontitis.

In the study described here, we determined the degree to which enhanced TNF levels in animals with type 2 diabetes contribute to compromised resolution of periodontal disease. The results demonstrate that diabetes-enhanced TNF plays an important role in the prolonged inflammation and osteoclastogenesis observed in type 2 diabetes in animals and that the enhanced inflammation through TNF significantly reduces the normal capacity to repair resorbed bone by suppressing the expression of growth factors that are needed to simulate proliferation and differentiation and inhibit apoptosis of osteoblasts or their precursors. These studies, for the first time, suggest a molecular basis by which type 2 diabetes could negatively affect bone in a number of pathological conditions by suppressing the expression of mediators needed for bone formation through enhanced and prolonged inflammation.

MATERIALS AND METHODS

Induction of periodontal bone loss and preparation of specimens

Male type 2 diabetes model Goto-Kakizaki (GK) and normoglycemic Wistar rats (8 wk old) were purchased from Charles River Laboratories (Wilmington, MA, USA). GK rats typically become diabetic at 8 wk of age, and experiments were started at 12 wk. Periodontitis was induced by tying silk ligatures around the maxillary second molars. Placement of ligatures induces periodontal disease by facilitating bacterial invasion of gingiva (20). After 7 d, ligatures were removed to initiate resolution of periodontal inflammation and a repair process. Rats were euthanized on d 0, 7 (ligature removal), 11, 15, and 22, based on a previous study (21). The maxillary and mandibular jaws were fixed in 4% paraformaldehyde for 3 d at 4°C and decalcified in Immunocal (Decal Chemical Corp., Tallman, NY, USA) at 4°C for 12 d. Sagittal paraffin sections were prepared at 5 μm; the area between the first and second molar was examined. Measurements in the gingival connective tissue were performed from the epithelial attachment to the crest of bone; measurements associated with bone were carried out from the crest of bone to a depth of 1 mm. A given data set was obtained by counting immunopostive cells or by histomorphometric analysis by one examiner who was calibrated by a second examiner. A diagram of the periodontal tissues is shown in Supplemental Fig. S1. All animal procedures were approved by the University of Medicine and Dentistry of New Jersey Institutional Animal Care and Use Committee.

Inhibition of TNF-α

Diabetic animals were treated with a TNF-α specific inhibitor, pegsunercept, that was generously provided by Amgen (Thousand Oaks, CA, USA). It was administered by intraperitoneal injection (4 mg/kg body weight) at the time of ligature removal and every 4 d thereafter in order to focus on the resolution of periodontal inflammation. Control animals were treated with vehicle alone following the same schedule. Serum glucose levels for vehicle- and pegsunercept-treated diabetic animals were similar, >400 mg/dl.

Detection of number and depth of polymorphonuclear (PMN) and mononuclear leukocytes

The number of mononuclear and PMN leukocytes and the depth of PMN cells in gingival connective tissue were counted in hematoxylin and eosin (H&E) stained sections examined at ×1000 view. The identification of these cells was confirmed by a pathologist.

Osteoclasts, osteoblasts, and osteoid and new bone formation

Osteoclasts were identified and counted as multinucleated tartrate-resistant acid phosphatase (TRAP)-positive bone-lining cells from the bone crest to a depth of 1.0 mm, as we have previously described (21). Osteoblasts were counted as cuboidal bone-lining cells in areas of bone remodeling, and their identity was confirmed by immunohistochemistry using an antibody specific for osteocalcin (data not shown). New bone formation was identified in TRAP-stained sections using the reversal line as a guide, as we have previously described (21). The area of osteoid formation was measured as the unmineralized bone matrix between osteoblasts and the mineralized bone surface evident in H&E-stained sections.

Detection of apoptotic bone-lining cells

Apoptotic cells were detected by an *in situ* transferase-mediated dUTP nick-end labeling (TUNEL) assay by means of the DeadEnd Fluorometric TUNEL System kit purchased from

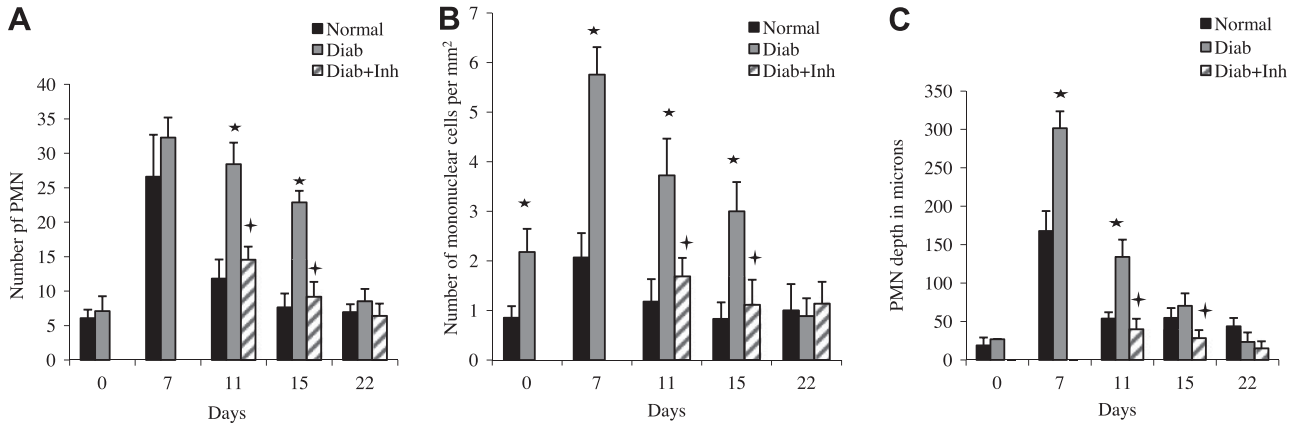


Figure 1. Diabetes prolongs an inflammatory infiltrate. Destructive periodontitis was initiated in rats by placement of ligatures around the molar teeth, which facilitates bacterial invasion of gingiva. Resolution of periodontal inflammation was induced by removal of ligatures. TNF was inhibited in diabetic rats by injection with pegsunercept when ligatures were removed and every 4 d thereafter (diab+inh group). Control rats received vehicle alone. Rats were euthanized before placing the ligature (0 d), simultaneously with ligature removal (7 d), and after ligature removal (11, 15, and 22 d). Numbers of PMN cells (*A*) and mononuclear cells (*B*) and depth of PMN cells (*C*) were examined in the gingival connective tissue from the epithelium adjacent to the tooth to distance of 1 mm toward alveolar bone, assessed in H&E-stained sections. $*P < 0.05$ *vs.* normoglycemic (normal) group; $^{+}P < 0.05$ *vs.* diabetic (diab) group.

Promega (Madison, WI, USA) with rTdT enzyme, following the manufacturer's instructions. The number of apoptotic cells was counted from the bone crest to a depth of 1 mm by computer-assisted analysis (Nikon, Melville, NY, USA) from images captured at ×200 view on an immunofluorescence microscope.

Immunohistochemistry

The numbers of cells expressing proliferating cell nuclear antigen (PCNA), bone morphogenetic protein-2 (BMP-2), and fibroblast growth factor-2 (FGF-2) either lining bone or adjacent to bone in the periodontal ligament (PDL) were counted. Sections were stained by immunohistochemistry using paraffin sections with an antibody specific for PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BMP-2 (NovusBiologicals, Littleton, CO, USA), or FGF-2 (Santa Cruz Biotechnology). Primary antibody was detected by avidin-biotin horseradish peroxidase complex using a biotinylated secondary antibody. To enhance the signal to noise ratio, citrate (pH 6) antigen retrieval was used along with tyramide signal amplification that enhances the chromogenic signal (PerkinElmer, Waltham, MA, USA). Sections were examined at ×600 view.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from periodontum (tooth, gingival, and alveolar bone around second and third molars) of vehicle- and pegsunercept-treated diabetic rats and assessed for mRNA of rat IFN-γ, TNF-α, IL-1β, FGF-2, transforming growth factor β-1 (TGFβ-1), BMP-2, and BMP-6 by real-time PCR using *Taq*-Man primers and probe sets (Applied Biosystems, Foster City, CA, USA). Results were normalized to a housekeeping gene, ribosomal protein L32. The experiments were carried out with 4 to 5 animals/group with triplicate samples and performed 2 to 3 times with similar results.

Statistical analysis

One-way analysis of variance was used to determine the differences between groups at a given time point and to compare difference from baseline values within a group. The significance level was set at $P < 0.05$.

RESULTS

To characterize the inflammatory response at the onset of periodontitis and during its resolution, the number of PMN cells was examined (**Fig. 1*A*** and Supplemental Fig. S2). On d 0 in the normoglycemic group, the number of PMN cells was low and increased 4.4-fold after the initiation of periodontitis (d 7; $P<0.05$). At later time points after ligatures were removed, the number of PMN cells decreased substantially and was close to baseline 8 d later (on d 15). The diabetic group at baseline was similar to the normoglycemic group and

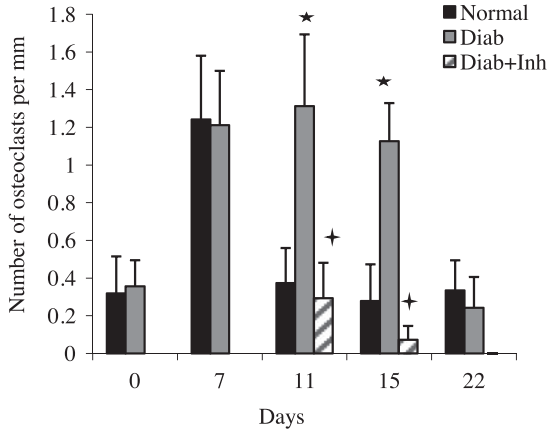


Figure 2. Diabetes increases osteoclast formation, which is due to prolonged inflammation. Induction and resolution of periodontal disease and application of TNF inhibitor were performed as in Fig. 1. Osteoclasts were counted in TRAP-stained sections as described in Materials and Methods. $*P < 0.05$ *vs.* normoglycemic group; $^{+}P < 0.05$ *vs.* diabetic group.

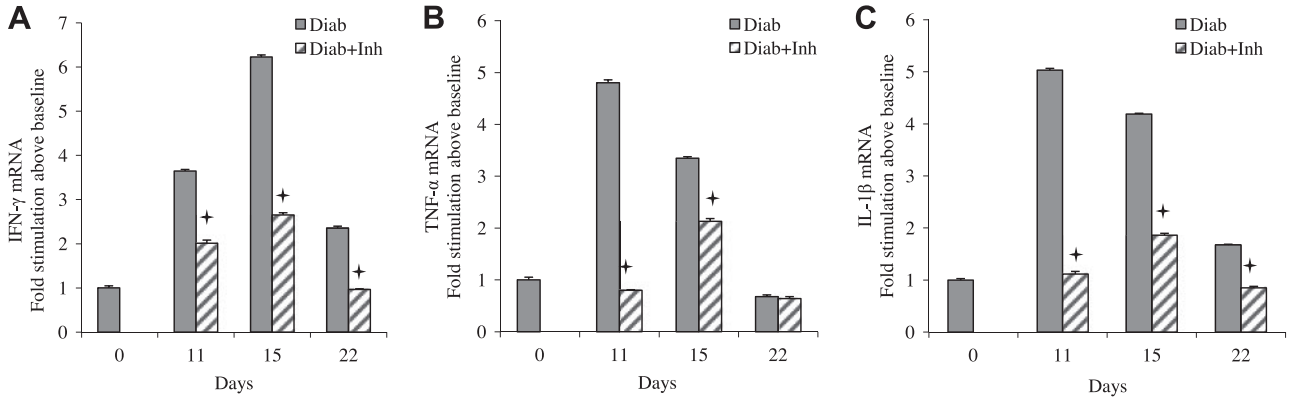


Figure 3. Diabetes prolongs mRNA levels of inflammatory cytokines. Induction and resolution of periodontitis and application of TNF inhibitor were performed as in Fig. 1. IFN-γ (*A*), TNF-α (*B*), and IL-1β (*C*) mRNA levels were measured in RNA extracted from rat periodontium by RT-PCR. $^{+}P < 0.05$ *vs.* diabetic group.

also increased 4.5-fold after the initiation of periodontitis. However, on d 11 and 15, the number of PMN cells was still high, so that the values were 2.4- and 3-fold higher in the diabetic group than in the normoglycemic group ($P<0.05$). By d 15, the numbers were similar to baseline values. Treatment of diabetic animals with a TNF inhibitor reduced the number of PMN cells to that of the normoglycemic animals. The number of mononuclear leukocytes followed a similar pattern, with high levels persisting in the diabetic group following removal of etiologic factors, which was normalized by treatment with a TNF-inhibitor (Fig. 1*B*).

Periodontal bone resorption has been linked to the transition of an inflammatory infiltrate from the subepithelial space to deeper areas of connective tissue. At baseline, PMN cells in both normoglycemic and diabetic groups were found only in proximity to the epithelium (Fig. 1*C*). The inflammatory infiltrate moved deeper within the connective tissue in both normoglycemic and diabetic groups with the onset of periodontitis, although considerably more so in the diabetic group, on d 7. On d 11, the depth of the infiltrate was reduced in the normoglycemic animals, while it remained substantially deeper in the diabetic group ($P<0.05$). Inhibition of TNF resulted in rapid reversal, so that the inflammatory infiltrate was restored to the subepithelial connective tissue area.

To establish how type 2 diabetes may affect the cessation of bone loss, osteoclast numbers were measured (**Fig. 2**). At baseline, the number of osteoclasts per millimeter of bone length was low in diabetic and normoglycemic groups. It increased 4-fold on d 7 in the normoglycemic group ($P<0.05$) and quickly returned to baseline levels within 4 d after the etiologic factor was removed (on d 11). Osteoclast numbers also increased in the diabetic rats with the initiation of periodontal disease but were 2.7- and 4-fold higher in the diabetic group *vs.* the normoglycemic group on d 11 and 15 ($P<0.05$). When treated with a TNF inhibitor, the number of osteoclasts in the diabetic group returned to normoglycemic levels.

Both the adaptive and innate immune responses are thought to contribute to periodontitis. To assess the effect of the TNF inhibitor on inflammatory cytokine mRNA levels in the periodontal tissue of diabetic rats, IFN-γ (**Fig. 3*A***), TNF-α (Fig. 3*B*), and IL-1β (Fig. 3*C*) were measured. IFN-γ mRNA levels in the diabetic animals were substantially higher during the resolution phase of periodontal inflammation compared to baseline. Similarly, IL-1 and TNF-α levels in the diabetic group were higher, and both were decreased by inhibition of TNF. The results indicate that the overall mRNA levels of inflammatory cytokines during the

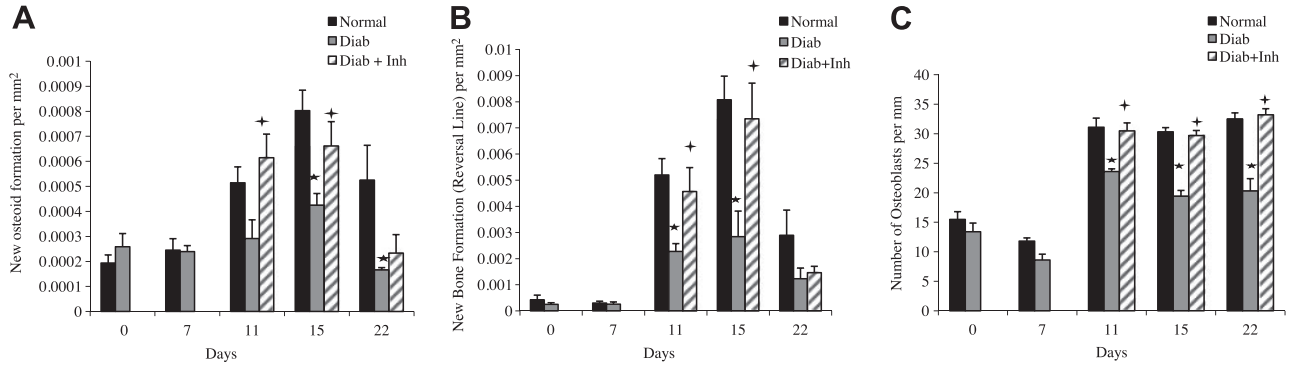


Figure 4. Diabetes reduces periodontal bone formation and osteoblast numbers, and this decrease is reversed in diabetic animals by TNF inhibition. New osteoid formation (*A*) and bone formation (*B*) were measured in histological sections; osteoblasts were counted per millimeter of bone length (*C*) in H&E-stained sections. Osteoblast counts were confirmed using osteocalcin-specific immunostaining. $^{*}P < 0.05$ *vs.* normoglycemic group; $^{+}P < 0.05$ *vs.* diabetic group.

resolution phase in the diabetic animals were reversed by inhibiting TNF.

Bone is programmed to repair, a process called coupled bone formation, which limits net bone loss following an episode of bone resorption. We examined the effect of type 2 diabetes on formation of osteoid, an immature bone matrix (**Fig. 4*A***), new bone formation (Fig. 4*B*), and the number of osteoblasts capable of forming bone matrix (Fig. 4*C*). The amounts of osteoid and new bone formation were low in both normoglycemic and diabetic groups at baseline and during the initiation of periodontal inflammation. When periodontal disease ceased, new osteoid formation increased 4-fold and bone formation >10-fold in the normoglycemic group, peaking 8 d after ligatures were removed (on d 15; $P<0.05$). The diabetic group showed a different pattern, without a significant burst of osteoid or bone formation. Thus, the formation of bone and osteoid was significantly less in the diabetic compared to normoglycemic animals ($P<0.05$). When treated with a TNF inhibitor, both osteoid and new bone formation in the diabetic group increased to a level equivalent to that of the normoglycemic group, which indicated that diabetes exerted its negative effect on bone formation largely through its effect on inflammation involving TNF.

A potential mechanism by which the amount of osteoid and new bone formation is limited by type 2 diabetes is the number of osteoblastic cells that can form bone matrix. This value increased >2.7-fold in the normoglycemic group after periodontal inflammation was reduced during the resolution of inflammation ($P<0.05$; Fig. 4*C*). The diabetic group had significantly fewer numbers of osteoblasts at each of these time points ($P<0.05$). The decrease in osteoblasts in the diabetic group was directly attributable to inflammation, as the number increased to normoglycemic levels when diabetic rats were treated with a TNF inhibitor.

Potential mechanisms by which the number of osteoblasts is limited in animals with type 2 diabetes include increased apoptosis (**Fig. 5*A***) and decreased proliferation of bone-lining cells (Fig. 5*B*). The number of apoptotic cells in the normoglycemic group increased 10-fold with the induction of periodontal disease and decreased during resolution of periodontal inflammation. The diabetic group exhibited a different pattern, so that the number of apoptotic bone-lining cells was typically 2-fold higher in the diabetic group compared to normoglycemic animals during the resolution phase. When treated with a TNF inhibitor, the higher level of apoptosis was reversed in diabetic animals and followed the same pattern as normoglycemic animals. Proliferation of bone-lining cells was also examined by immunohistochemistry for expression of PCNA (Supplemental Fig. S3). The level of proliferating bone-lining cells significantly increased during the resolution phase in normoglycemic but not in diabetic rats. When TNF was inhibited in diabetic animals, the number of cells expressing PCNA was restored to normoglycemic levels. PCNA-immunopositive cells in proximity to bone were predominantly fibroblastic (**Table 1**). However, the proportion of fibroblasts and bone-lining cells that were immunopositive for PCNA were similar.

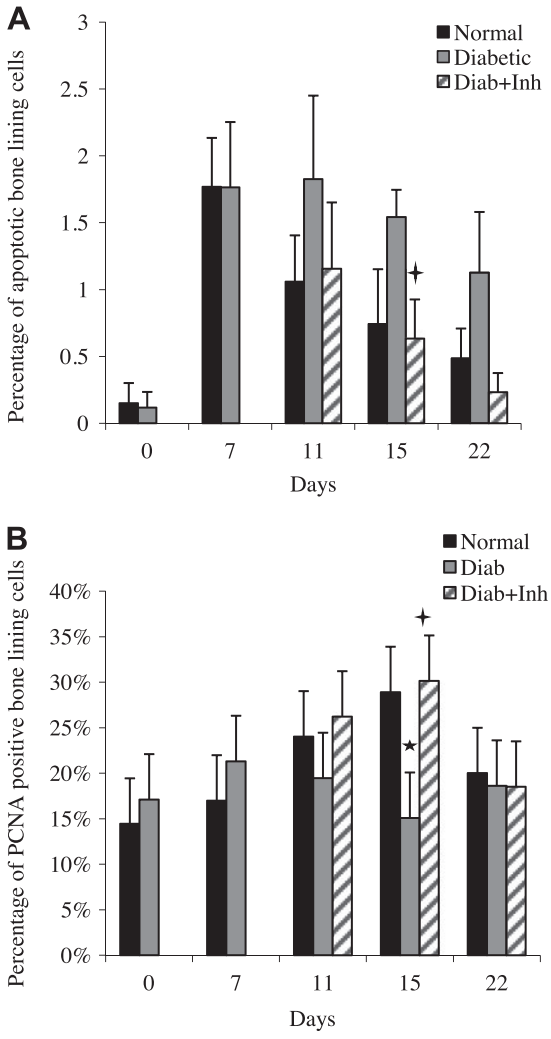


Figure 5. Diabetic rats have increased apoptosis and decreased proliferation of bone-lining cells, which is reversed by TNF inhibition. Induction and resolution of periodontal disease and application of TNF inhibitor were performed as in Fig. 1. *A*) Apoptotic bone-lining cells in histological sections were measured by the TUNEL assay. *B*) Number of PCNA-immunopositive cells was measured by immunohistochemistry. $^{*}P < 0.05$ *vs.* normoglycemic group; $^{+}P < 0.05$ *vs.* diabetic group.

Apoptosis and proliferation of bone-lining cells may be modulated by growth factors that stimulate osteoblasts or their precursors. We examined mRNA levels of a number of factors that affect bone, including TGFβ-1 (**Fig. 6*A***), BMP-2 (Fig. 6*B*), BMP-6 (Fig. 6*C*), and FGF-2 (Fig. 6*D*), focusing on the period of resolution, when apoptosis was elevated and proliferation reduced in the diabetic group. During this period in diabetic animals, little or no increase was found in mRNA levels of these growth factors compared to baseline. However, when TNF was inhibited, mRNA for each of these growth factors increased, with peaks ranging from 2- to 6-fold.

To determine whether there was increased expression of growth factors in proximity to bone, immunohistochemistry was performed, measuring the number of BMP-2-immunopositive cells (**Fig. 7*A*** and Supplemental Fig. S4) and FGF-2-immunopositive cells (Fig. 7*B*) in the periodontal ligament adjacent to bone. At baseline and at

TABLE 1. *Immunopositive cells that expressed PCNA, BMP-2, and FGF-2*

Expression	Relative ranking	
	Based on total number of cells	Based on proportion
PCNA	Fibroblasts > bone-lining cells > endothelial cells	Fibroblasts = bone-lining cells > endothelial cells
BMP-2	Fibroblasts > bone-lining cells > endothelial cells	Bone-lining cells > fibroblasts > endothelial cells
FGF-2	Fibroblasts > bone-lining cells > endothelial cells	Fibroblasts = bone-lining cells > endothelial cells

Immunopositive cells in proximity to bone were assessed as bone-lining, fibroblastic, or endothelial cells that were part of blood vessels.

the onset of periodontal disease, the number of BMP-2-immunopositive cells was low and similar in the normoglycemic and diabetic groups. BMP-2 expression increased almost 2-fold in the normoglycemic group when periodontal disease was halted but exhibited little increase in the diabetic group. However, when TNF was inhibited, a 2-fold increase was found in the number of BMP-2-immunopositive cells ($P<0.05$). A similar pattern was observed for FGF-2. In the normoglycemic group, the number of FGF-2-positive cells increased after ligature removal, but relatively little change was found in the diabetic animals. When diabetic animals were treated with TNF inhibitor, a significant 1.7-fold increase was found in the number of FGF-2-expressing cells. BMP-2- and FGF-2-immunopositive cells in proximity to bone were primarily fibroblasts (Table 1). Other cells that prominently expressed BMP-2 and FGF-2 were bone-lining and endothelial cells.

DISCUSSION

Results described here provide new insight as to how type 2 diabetes can affect bone by its effect on the resolution of inflammation. It is well known that the inflammatory response, rather than the direct pathological effects of bacteria, is pivotal in stimulating periodontal disease (4). Bacteria stimulate an inflammatory response that induces a series of changes that can be damaging to the periodontal tissue, including the destruction of connective tissue matrix and resorption of bone. We found that the formation of a PMN or mononuclear cell inflammatory infiltrate in the type 2 diabetes model rats peaked on d 7 after the initiation of periodontitis and that this inflammation was prolonged compared to the normoglycemic rats, which was normalized by treatment with a TNF inhibitor. Inhibition of TNF also reversed the expression of proinflammatory cytokines induced by initiation of periodontitis. The TNF inhibitor caused a general reduction in cytokine levels; cytokines associated with both the innate and adaptive immune response were reduced significantly when TNF was inhibited. The consequence of these inflammatory changes was a prolonged period of high osteoclast numbers, which was reversed when TNF was blocked. TNF antagonists can directly or indirectly regulate differentiation and activation of osteoclasts, thus reducing bone destruction under pathological conditions

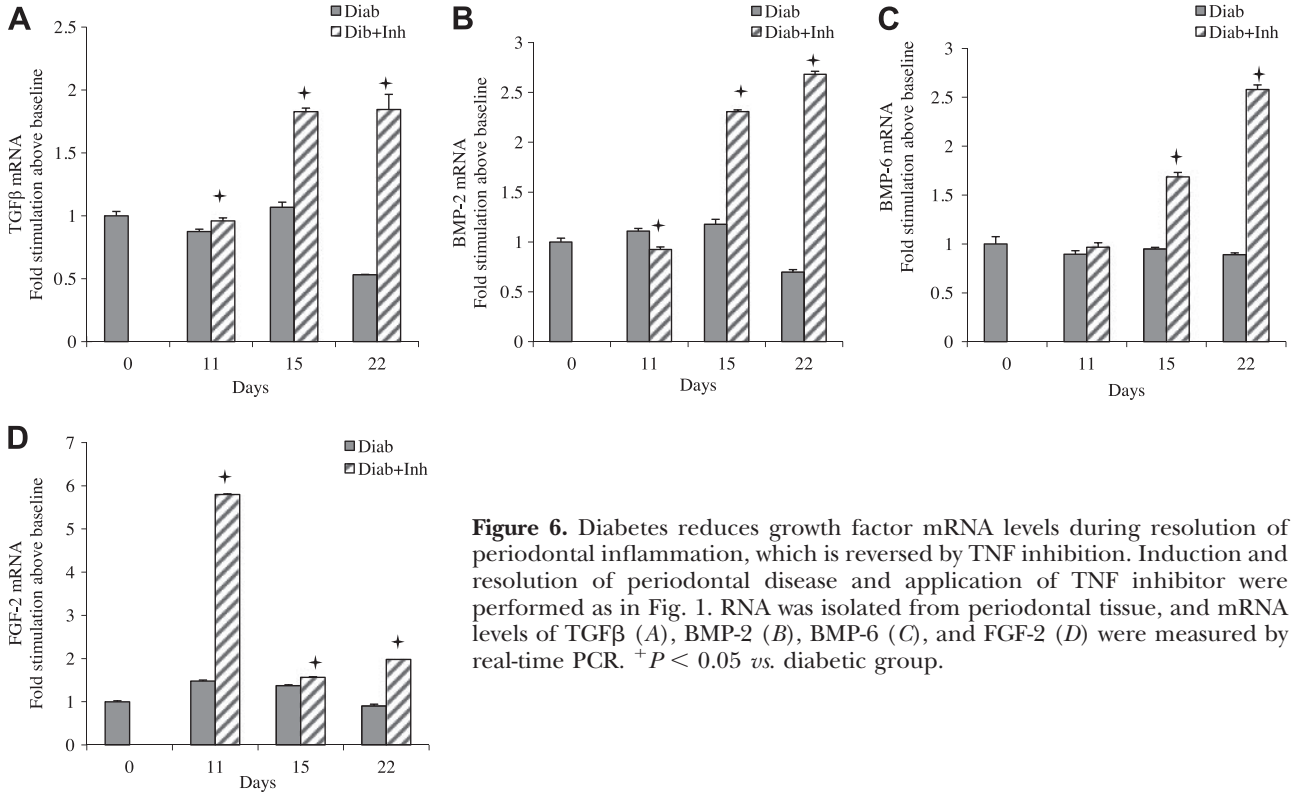


Figure 6. Diabetes reduces growth factor mRNA levels during resolution of periodontal inflammation, which is reversed by TNF inhibition. Induction and resolution of periodontal disease and application of TNF inhibitor were performed as in Fig. 1. RNA was isolated from periodontal tissue, and mRNA levels of TGFβ (*A*), BMP-2 (*B*), BMP-6 (*C*), and FGF-2 (*D*) were measured by real-time PCR. $^{+}P < 0.05$ *vs.* diabetic group.

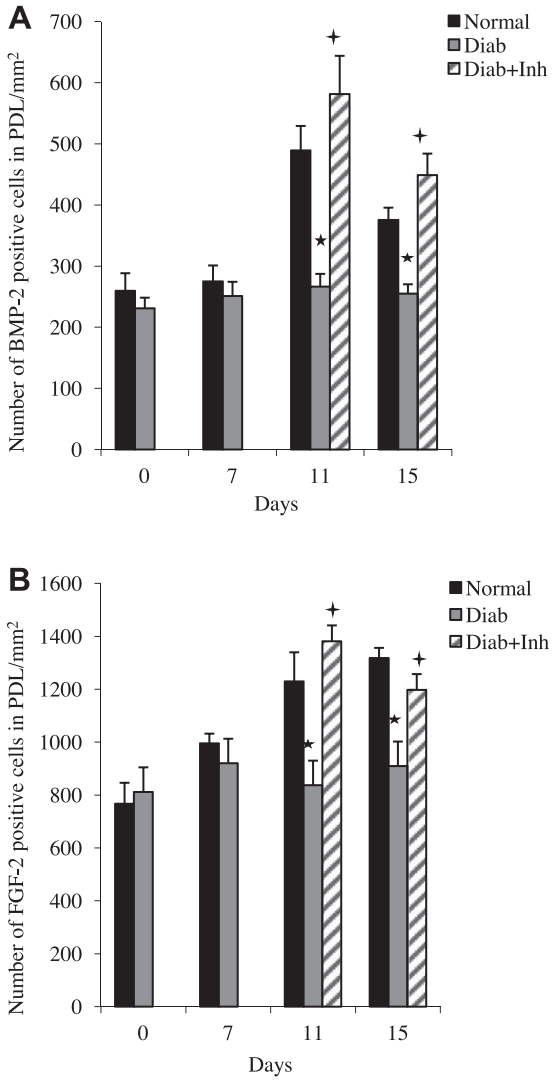


Figure 7. TNF inhibition enhances BMP-2 and FGF-2 production in the periodontium in diabetic specimens. Induction and resolution of periodontal disease and application of TNF inhibitor were performed as in Fig. 1. Numbers of BMP-2- and FGF-2-expressing cells in proximity to bone (within the periodontal ligament) were measured by immunohistochemistry using antibodies specific for BMP2 (*A*) and FGF-2 (*B*). $*P < 0.05$ *vs.* normoglycemic group; $^{+}P < 0.05$ *vs.* diabetic group.

(22). Thus, a consequence of prolonged inflammation in the diabetic periodontium is a difficulty in turning off osteoclasts and bone resorption, leading to a longer period of periodontal destruction.

Bone resorption is followed by a period of bone formation, a coupling process that limits the amount of net bone loss, which occurs during the resolution of inflammation in the periodontium (4). We found that type 2 diabetes model GK rats do not generate a burst of bone formation during resolution of inflammation, agreeing with results previously shown in a different type 2 diabetes model, the Zucker diabetic fatty rat (21). Although it has been known that decreased bone formation occurs in diabetic animals, the relationship between inflammation and diminished bone formation has not previously been established. Results presented here demonstrate that the reversal of inflammation increases the capacity of the animal with type 2 diabetes to form new bone. The molecular mechanisms for this reversal were investigated by examining the effect of type 2 diabetes on the production of growth factors that control proliferation, differentiation, or apoptosis of osteoblasts or their precursors. The expression of these factors was blunted in animals with type 2 diabetes but was significantly reversed when inflammation was resolved by inhibition of TNF. The results thus directly link prolonged inflammation in the diabetic group with impaired expression of bone-regulating growth factors. It is also striking that when inflammation was resolved in the diabetic animals by treatment with pegsunercept, substantial improvement was found in the cellular events regulated by these factors, including an increase in proliferation and formation of osteoblasts and a decrease in apoptosis of bone-lining cells.

Previous studies have examined the effect of TNF-specific inhibitors on bone formation. Humans treated with infliximab, a monoclonal antibody against TNF-α, had increased osteocalcin, a marker of bone formation in rheumatoid arthritis, osteoporosis, and other conditions (23). Moreover, infliximab therapy in Crohn's disease influences bone metabolism by enhancing bone formation and decreasing bone resorption. TNF impairs the function of bone-forming osteoblasts by suppressing mature osteoblast function, such as the production of a matrix that is competent for mineralization and by blocking the differentiation of osteoblasts where inflammation is thought to be present (24). This finding is consistent with reports that TNF inhibits differentiation of osteoblasts *in vitro* (25, 26) and also interferes with bone morphogenetic protein signaling (27). Although it has been reported that inflammation limits bone formation in osteoporosis by reducing Fra-1, we did not observe changes in Fra-1 mRNA levels (data not shown and ref. 28). It has also been reported that the anti-inflammatory mediator, resolvin E1, promotes regeneration of periodontal tissue, which may be related to its anti-inflammatory properties (8, 29). The experiments presented in the present study represent proof of principle that excessive production of inflammatory mediators such as TNF-α limits bone coupling in periodontitis. However, the experimental strategy may not necessarily be extrapolated directly to human studies due to the importance of TNF in up-regulating antibacterial defenses (30, 31).

In sumary, diabetes has an important effect on the periodontium. Both type 1 and type 2 diabetes model animals exhibit an increase in TNF-α in response to a bacterial stimulus when compared to normoglycemic controls (14, 32).We examined the effect of diabetes during the resolution of periodontal inflammation and found that type 2 diabetes prolongs enhanced inflammation. The ramifications of this prolonged inflammation were studied by examining the impact on bone. The diabetic condition reduced coupled bone formation that occurred in the normoglycemic group during resolution of periodontal inflammation. This finding was linked directly to inflammation, as the amounts of new bone and osteoid formed were reversed by inhibiting TNF. The effect was not due to the changes in hyperglycemia, as serum glucose levels did not significantly change after treatment with pegsunercept. The mechanism by which

inflammation affects bone was studied by examining factors that regulate bone cells. The production of these factors was enhanced significantly when inflammation was reduced in diabetic animals and explains the increased proliferation and reduced apoptosis of bone cells, as well as the increased numbers of osteoblasts. These results provide a mechanistic basis for how diabetes can negatively affect bone through the effects of enhanced inflammation on the expression of critical factors needed to stimulate bone formation. FJ

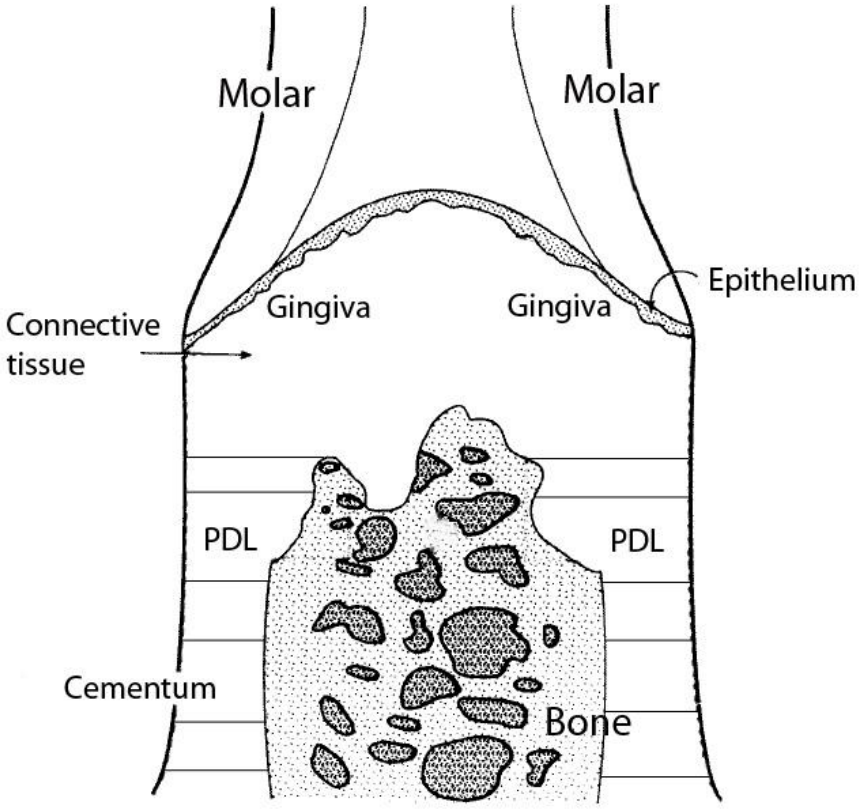
The authors thank Tesfahun Desta (University of Medicine and Dentistry of New Jersey) for help in the experiments and Sunitha Batchu (University of Pennsylvania School of Dental Medicine) for help in preparing the manuscript. This work was supported by the U.S. National Institute of Dental and Craniofacial Research, grants DE018307 and DE0DE17732.

REFERENCES

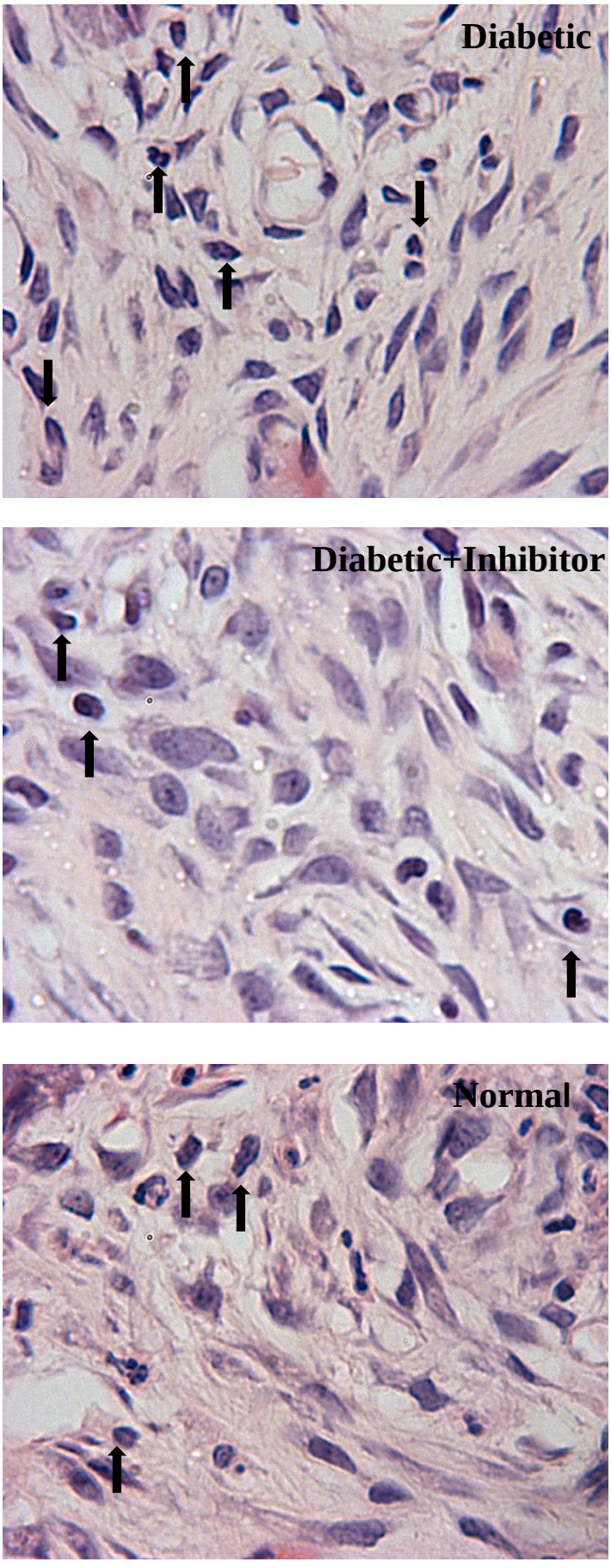
1. Graves, D. T., and Kayal, R. A. (2008) Diabetic complications and dysregulated innate immunity. *Front. Biosci.* **13,** 1227–1239
2. Lamster, I. B., Lalla, E., Borgnakke, W. S., and Taylor, G. W. (2008) The relationship between oral health and diabetes mellitus. *J. Am. Dent. Assoc.* **139**(Suppl.), 19S–24S
3. Kumar, P. S., Leys, E. J., Bryk, J. M., Martinez, F. J., Moeschberger, M. L., and Griffen, A. L. (2006) Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J. Clin. Microbiol.* **44,** 3665–3673
4. Graves, D. T., Li, J., and Cochran, D. L. (2010) Inflammation and uncoupling as mechanisms of periodontal bone loss. *J. Dent. Res.* **90,** 143–153
5. Williams, R., Jeffcoat, M., Kaplan, M., Goldhaber, P., Johnson, H., and Wechter, W. (1985) Flurbiprofen: a potent inhibitor of alveolar bone resorption in beagles. *Science* **227,** 640–642
6. Assuma, R., Oates, T., Cochran, D., Amar, S., and Graves, D. (1998) IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *J. Immun.* **160,** 403–409
7. Yu, H., Li, Q., Herbert, B., Zinna, R., Martin, K., Junior, C. R., and Kirkwood, K. L. (2011) Anti-inflammatory effect of MAPK phosphatase-1 local gene transfer in inflammatory bone loss. *Gene Ther.* **18,** 344–353
8. Hasturk, H., Kantarci, A., Ohira, T., Arita, M., Ebrahimi, N., Chiang, N., Petasis, N. A., Levy, B. D., Serhan, C. N., and Van Dyke, T. E. (2006) RvE1 protects from local inflammation and osteoclast- mediated bone destruction in periodontitis. *FASEB J.* **20,** 401–403
9. Garlet, G. P. (2010) Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *J. Dent. Res.* **89,** 1349–1363
10. Baker, P., Dixon, M., Evans, R., Dufour, L., Johnson, E., and Roopenian, D. (1999) CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. *Infect. Immun.* **67,** 2804–2809
11. Delima, A., Spyros, K., Amar, S., and Graves, D. T. (2002) Inflammation and tissue loss caused by periodontal pathogens is reduced by IL-1 antagonists. *J. Infect. Dis.* **186,** 511–516
12. Teng, Y., Nguyen, H., Gao, X., Kong, Y., Gorczynski, R., Singh, B., Ellen, R., and Penninger, J. (2000) Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J. Clin. Invest.* **106,** R59–67
13. Han, X., Kawai, T., Eastcott, J. W., and Taubman, M. A. (2006) Bacterial-responsive B lymphocytes induce periodontal bone resorption. *J. Immunol.* **176,** 625–631
14. Naguib, G., Al-Mashat, H., Desta, T., and Graves, D. (2004) Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. *J. Invest. Dermatol.* **123,** 87–92
15. Spite, M., and Serhan, C. N. (2010) Novel lipid mediators promote resolution of acute inflammation: impact of aspirin and statins. *Circ. Res.* **107,** 1170–1184
16. Salvi, G., Collins, J., Yalda, B., Arnold, R., Lang, N., and Offenbacher, S. (1997) Monocytic TNFalpha secretion patterns in IDDM patients with periodontal diseases. *J. Clin. Periodontol.* **24,** 8–16
17. Graves, D., Oskoui, M., Volejnikova, S., Naguib, G., Cai, S., Desta, T., Kakouras, A., and Jiang, Y. (2001) Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to P. gingivalis infection. *J. Dent. Res.* **80,** 1875–1879
18. Garlet, G. P., Cardoso, C. R., Campanelli, A. P., Ferreira, B. R., Avila-Campos, M. J., Cunha, F. Q., and Silva, J. S. (2007) The dual role of p55 tumour necrosis factor-alpha receptor in Actinobacillus actinomycetemcomitans-induced experimental periodontitis: host protection and tissue destruction. *Clin. Exp. Immunol.* **147,** 128–138
19. Lalla, E., Lamster, I. B., Feit, M., Huang, L., Spessot, A., Qu, W., Kislinger, T., Lu, Y., Stern, D. M., and Schmidt, A. M. (2000) Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. *J. Clin. Invest.* **105,** 1117–1124
20. Rovin, S., Costich, E. R., and Gordon, H. A. (1966) The influence of bacteria and irritation in the initiation of periodontal disease in germfree and conventional rats. *J. Periodontal Res.* **1,** 193–204
21. Liu, R., Bal, H. S., Desta, T., Krothapalli, N., Alyassi, M., Luan, Q., and Graves, D. T. (2006) Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. *J. Dent. Res.* **85,** 510–514
22. Fantuzzi, F., Del Giglio, M., Gisondi, P., and Girolomoni, G. (2008) Targeting tumor necrosis factor alpha in psoriasis and psoriatic arthritis. *Expert Opin. Ther. Targets* **12,** 1085–1096
23. Miheller, P., Muzes, G., Racz, K., Blazovits, A., Lakatos, P., Herszenyi, L., and Tulassay, Z. (2007) Changes of OPG and RANKL concentrations in Crohn's disease after infliximab therapy. *Inflamm. Bowel Dis.* **13,** 1379–1384
24. Pacifici, R. (2010) The immune system and bone. *Arch. Biochem. Biophys.* **503,** 41–53
25. Gilbert, L., He, X., Farmer, P., Boden, S., Kozlowski, M., Rubin, J., and Nanes, M. S. (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* **141,** 3956–3964
26. Lencel, P., Delplace, S., Hardouin, P., and Magne, D. (2011) TNF-alpha stimulates alkaline phosphatase and mineralization through PPARgamma inhibition in human osteoblasts. *Bone* **48,** 242–249
27. Guo, R., Yamashita, M., Zhang, Q., Zhou, Q., Chen, D., Reynolds, D. G., Awad, H. A., Yanoso, L., Zhao, L., Schwarz, E. M., Zhang, Y. E., Boyce, B. F., and Xing, L. (2008) Ubiquitin ligase Smurf1 mediates tumor necrosis factor-induced systemic bone loss by promoting proteasomal degradation of bone morphogenetic signaling proteins. *J. Biol. Chem.* **283,** 23084–23092
28. Chang, J., Wang, Z., Tang, E., Fan, Z., McCauley, L., Franceschi, R., Guan, K., Krebsbach, P. H., and Wang, C. Y. (2009) Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat. Med.* **15,** 682–689
29. Hasturk, H., Kantarci, A., Goguet-Surmenian, E., Blackwood, A., Andry, C., Serhan, C. N., and Van Dyke, T. E. (2007) Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. *J. Immunol.* **179,** 7021–7029
30. Havell, E. A. (1989) Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J. Immunol.* **143,** 2894–2899
31. Kim, E. Y., Priatel, J. J., Teh, S. J., and Teh, H. S. (2006) TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. *J. Immunol.* **176,** 1026–1035
32. Graves, D. T., Naguib, G., Lu, H., Leone, C., Hsue, H., and Krall, E. (2005) Inflammation is more persistent in Type 1 diabetic mice. *J. Dent. Res.* **84,** 324–328

Received for publication September 6, 2011.
Accepted for publication December 5, 2011.

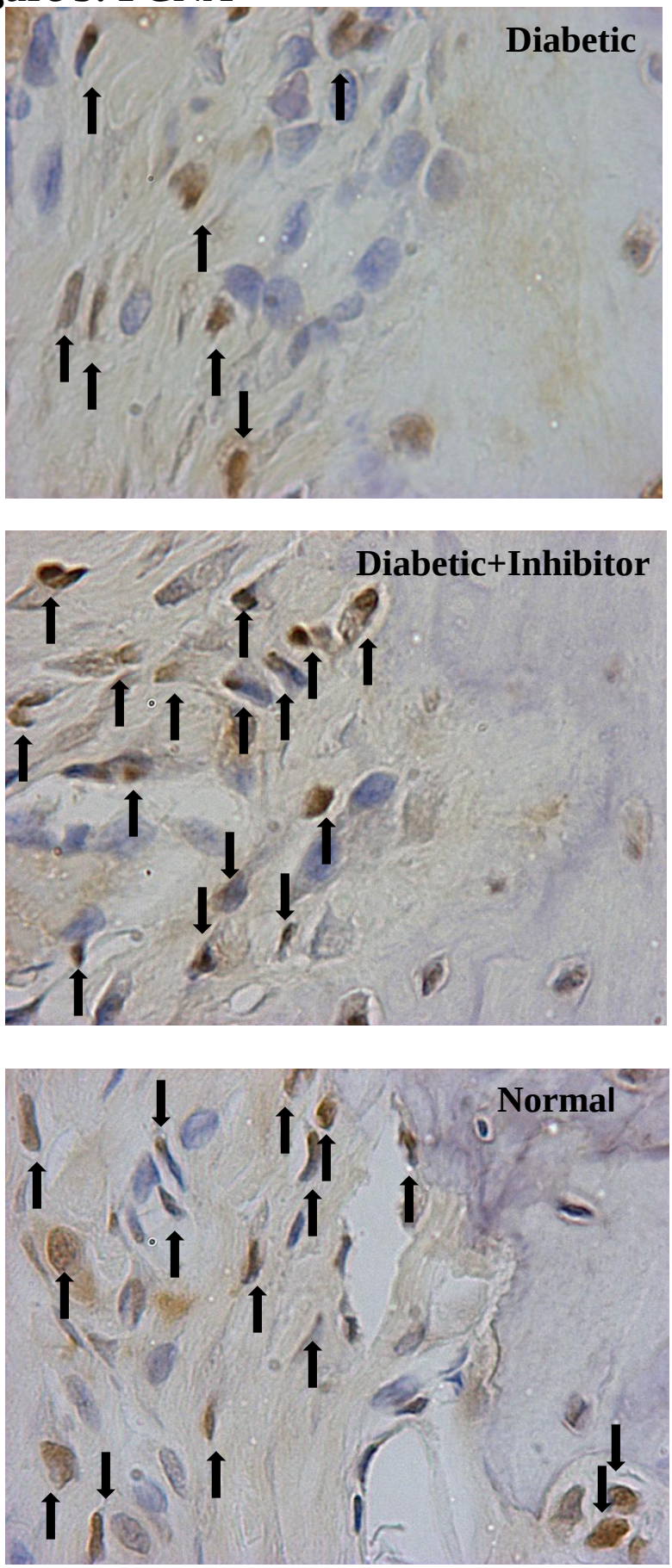
Supplemental figure 1



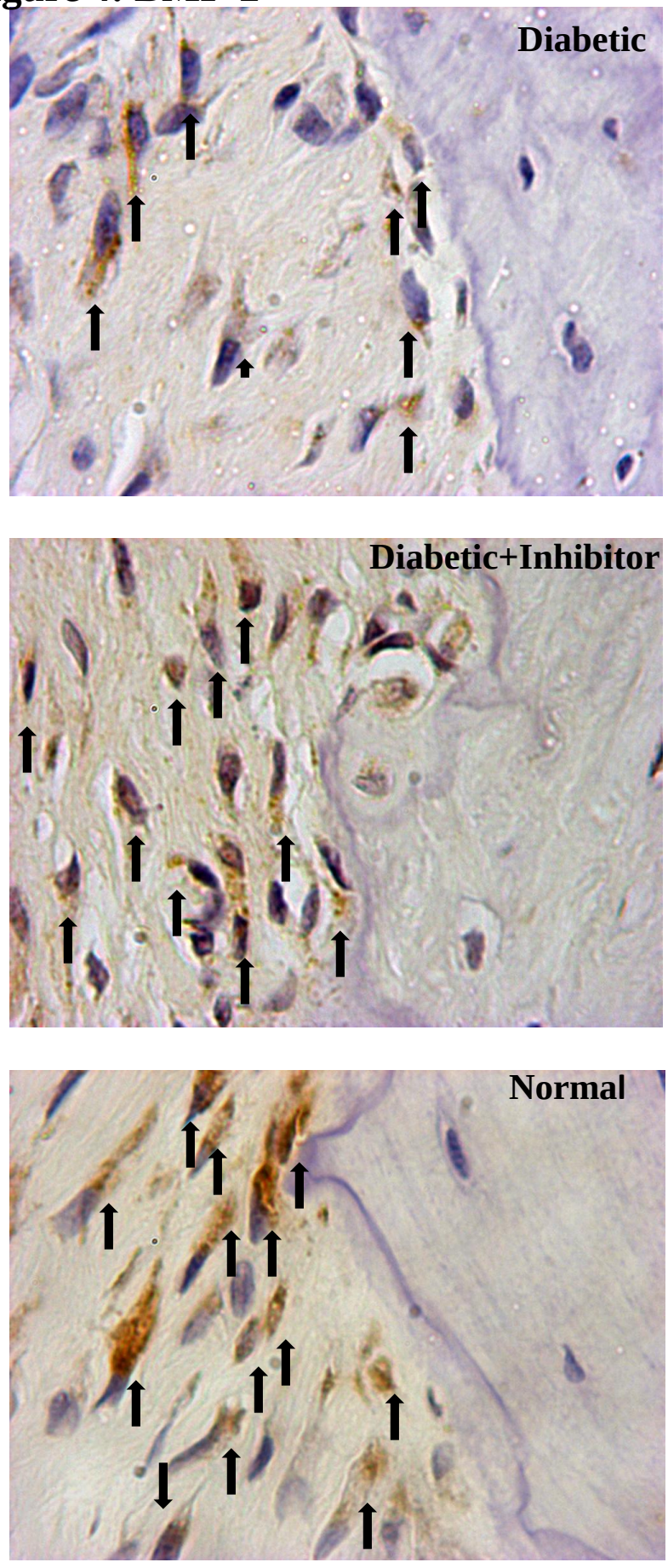
Supplemental figure 2



Supplemental figure 3: PCNA



Supplemental figure 4: BMP-2



FUTURE DIRECTIONS

FUTURE DIRECTIONS

1. **Testing whether apoptosis induced by periodontal infection significantly contributes to bone loss in diabetic animals.**

The functional role of apoptosis in pathologic processes can be studied with caspase inhibitors, which are small peptides that block the activity of well-defined caspases. These inhibitors have been used in animal models to attenuate cell death and diminish tissue damage in ischemic conditions, sepsis and other pathologic processes. Other studies using caspase inhibitors have shown that part of the detrimental effect of diabetes on healing after infection is due to increased fibroblast or osteoblast apoptosis. To understand how diabetes may affect periodontal bone loss through apoptosis we will use a caspase-3/7 inhibitor in a type 2 GK diabetic rat model of periodontal disease induced by bacterial infection. The aim of the study will be to determine how apoptosis of osteoblasts contributed to periodontal bone loss by its effect on bone formation in diabetic animals.

2. **Mechanisms by which we can get bone formation after periodontal bone loss.**

It has been well documented that inflammation plays a critical role in bacteria induced periodontal bone loss. However, the specific cell types in the role that each place has not been established. We will examine the role of osteoblast lineage cells by inducing periodontitis in wild type and transgenic mice to establish whether interfering with some inflammation pathways will affect in bone coupling.

3. **Reduce diabetes associated complications understand the patho-physiology of delayed wound closure in diabetes and identifying the pathways associated with accelerated wound repair.**

We have uncovered evidence implicating a set of molecules called Forkhead box, class O1 (FOXO1) in this process. FOXO is a family of transcription factors (proteins that control the expression of genes within the nucleus) that are normally turned off by insulin. We will examine the role of FOXO1 in wound healing in a diabetic transgenic mouse with specific FOXO1 deletion.

TRANSLATION

HIPÓTESIS

HIPÓTESIS

Hipótesis 1:

- **Hipótesis nula (H0):** la infección por *A. actinomycetemcomitans* en las ratas Wistar no aumenta la respuesta inflamatoria local y sistémica, la pérdida de inserción y el número de osteoclastos causando la pérdida de hueso periodontal.
- **Hipótesis alternativa (H1):** la infección por *A.actinomycetemcomitans* en la ratas Wistar aumenta la respuesta inflamatoria local y sistémica, la pérdida de inserción y el número de osteoclastos causando la pérdida de hueso periodontal.

Hipótesis 2:

- **Hipótesis nula (H0):** la inflamación en los animales diabéticos no dará lugar a mayores niveles de apoptosis que interferirán con la capacidad de reparar el hueso después de la pérdida ósea inducida.
- **Hipótesis alternativa (H1):** la inflamación en los animales diabéticos dará lugar a mayores niveles de apoptosis que interferirán con la capacidad de reparar el hueso después de la pérdida ósea inducida.

Hipótesis 3:

- **Hipótesis nula (H0):** la adición de un determinado agente bloqueante del TNF (pegsunercept) o inhibidor de la caspasa-3 no va a reducir el impacto de la diabetes ni mejorar la reparación ósea después de la resorción ósea periodontal.
- **Hipótesis alternativa (H1):** la adición de un determinado agente bloqueante del TNF (pegsunercept) o inhibidor de la caspasa-3 va a reducir el impacto de la diabetes y va a mejorar la reparación ósea después de la resorción ósea periodontal.

Hipótesis 4:

- **Hipótesis nula (H0):** la respuesta inflamatoria no disminuye en los animales diabéticos después de un tratamiento con antibióticos debido a la dificultad en disminuir la respuesta inflamatorias frente a una infección.
- **Hipótesis alternativa (H1):** la respuesta inflamatoria disminuye en los animales diabéticos después de un tratamiento con antibióticos debido a la dificultad en disminuir la respuesta inflamatorias frente a una infección.

Hipótesis 5:

- **Hipótesis nula (H0):** la formación de hueso mejorado no se reflejará en un aumento en el número de células osteoblásticas/mesenquimales que alinean el hueso.
- **Hipótesis alternativa (H1):** la formación ósea mejorada se refleja por un aumento en el número de células osteobásticas/mesenquimales que alinean el hueso.

OBJETIVOS

OBJETIVOS

Objetivo principal:

El objetivo del presente proyecto es evaluar el inicio y el desarrollo de la enfermedad periodontal y sus cambios en el desarrollo con la diabetes.

Objetivos secundarios:

1. Evaluar si la infección con *A. actinomycetemcomitans* es un buen modelo para estudiar el inicio y la progresión de la inflamación en la enfermedad periodontal y cómo se modifica con el tratamiento antibiótico.
2. Evaluar como la diabetes modifica el inicio y la progresión de la inflamación en la enfermedad periodontal y cómo se modifica con el tratamiento antibiótico.
3. Evaluar la resolución de la inflamación y cómo esta afecta en la reparación ósea.

CONCLUSIÓN

CONCLUSIÓN

1. Evaluar si la infección con *A. actinomycetemcomitans* es un buen modelo para estudiar el inicio y la progresión de la inflamación en la enfermedad periodontal y cómo se modifica con el tratamiento antibiótico.

En conclusión, se ha demostrado que las ratas Wistar son un buen modelo para observar los efectos de la bacteria *A. actinomycetemcomitans* que induce la enfermedad periodontal.

La infección con *A. actinomycetemcomitans* aumenta los niveles de células CD8+T en los ganglios linfáticos y los monocitos que se encuentran en la sangre periférica. La infección con *A. actinomycetemcomitans* induce la expresión de TNF-α en el epitelio de unión y provoca la pérdida de inserción. Esta pérdida de inserción fue revertida por el uso de tratamiento antibiótico dándonos una visión más clara de las vías de actuación de la enfermedad periodontal (Hipótesis 1).

2. Evaluar como la diabetes modifica el inicio y la progresión de la inflamación en la enfermedad periodontal y cómo se modifica con el tratamiento antibiótico.

Además, la diabetes aumenta la respuesta inflamatoria causada por *A. actinomycetemcomitans* con una excesiva producción de TNF-α en el tejido periodontal y una reabsorción alveolar ósea. Este último punto puede verse afectado por aumento de la apoptosis en el tejido periodontal como fue mostrado con el aumento de apoptosis en el epitelio gingival y en el tejido conectivo de las ratas (Hipótesis 2). El mecanismo en el que la apoptosis parece tener lugar es a través de la caspasa-3, ya que bloqueando la caspasa-3 disminuyó los niveles de apoptosis en el eptielio gingival y en el tejido conectivo de las ratas (Hipótesis 3).

Los antibióticos fueron capaces de invertir muchos parámetros de la respuesta local del huésped en animales diabéticos, pero no en los animales normoglucémicos. Esto podría indicar un déficit en la capacidad de los animales diabéticos para resistir una infección. Esta información proporciona una valiosa visión sobre cómo la diabetes puede alterar las

interacciones huésped-bacteria de forma que promueve la destrucción periodontal (Hipótesis 4).

3. Evaluar la resolución de la inflamación y cómo esta afecta en la reparación ósea.

Además, hemos examinado el efecto de la diabetes durante la resolución de la inflamación periodontal y hemos encontrado que la diabetes prolonga la inflamación. Las ratas diabéticas mostraron menor acoplamiento ósea en comparación con el grupo de ratas normoglucémicos. Este hallazgo se ligó a la inflamación, puesto que la inhibición de TNF invirtió las cantidades del número de osteoblastos, de nuevo hueso y osteoide (Hipótesis 5). Los mecanismos por los que la inflamación afecta al hueso se estudiaron mediante los factores que regulan las células óseas. Estos, fueron limitados por la producción de factores de crecimiento que controlan la proliferación y la apoptosis. Cuando TNF-α se inhibió, las parámetros de las ratas diabéticas mejoraron de una manera similar a las ratas normoglucémicas (Hipótesis 3).

Estos resultados proporcionan una explicación de cómo la diabetes puede afectar negativamente al hueso a través de los efectos de la inflamación en la expresión de los factores críticos necesarios para estimular la formación de hueso.

RESUMEN

RESUMEN

El objetivo de este estudio fue evaluar la respuesta histológica y celular a la infección por *A.actinomycetemcomitans (A.a)* y como la diabetes exacerba la producción de TNF- α y la apoptosis que contribuye a la progresión de la enfermedad periodontal y al acoplamiento del hueso.

Ratas Goto-Kakizaki (GK) y normoglucémicas (Wistar) fueron utilizadas en este estudio. La enfermedad periodontal fue inducida por la inoculación con *A. a* y por la colocación de una ligadura alrededor de los segundos molares superiores que facilita la invasión bacteriana de la encía. Las ratas que fueron inoculadas por *A a* fueron sacrificadas en diferentes grupos: antes de inocular la bacteria y 4, 5 y 6 semanas después. Las ratas a las que se les colocó la ligadura fueron sacrificadas el día 0, 7 (cuando se les sacó la ligadura), el día 11, el 15 y el 22. Varios parámetros fueron evaluados en los animales a los que se inoculó *A. a*: respuesta a los anticuerpos, colonización oral, pérdida ósea, pérdida de inserción, PMNs, TNF-α, células apoptóticas, osteoclastos y la respuesta inmune adaptativa en los ganglios linfáticos locales. A algunos grupos se les dio tratamiento antibiótico a las 4 semanas. En el estudio donde se colocó la ligadura se le aplicó un inhibidor específico del tumor de necrosis factor (TNF), pegsunrecept, en algunas ratas diabéticas después del inicio de a enfermedad periodontal. Se midieron factores de crecimiento con RT-PCR, y las secciones histológicas fueron examinadas para la infiltración de leucocitos y varios parámetros relacionados con la resorción y la formación ósea.

Una respuesta de anticuerpos contra *A. a* se produjo dentro de las 4 semanas de la infección. Además el 78% de las ratas inoculadas tenían *A. a* detectable en la cavidad oral ($p < 0.05$). La infección con *A. a* aumentó significativamente la pérdida de inserción que fue revertido por el tratamiento antibacteriano ($p < 0.05$). La expresión de TNF-α en el epitelio de unión siguió el mismo patrón. ***A. a*** estimuló la formación de osteoclastos y la expresión de TNF-α en el tejido conectivo ($p < 0.05$). El reclutamiento de PMNs aumentó significativamente después de la infección por ***A. a*** ($p < 0.05$). ***A. a*** también aumentó el número de células T CD8+ ($p < 0.05$), pero no las células T CD4+ ni las células T reguladoras (Tregs) ($p > 0.05$). Después de la infección con ***A. a***, la pérdida de hueso en las

ratas diabéticas y la infiltración de PMNs, fue 1.6 veces mayor comparado con las ratas normoglucémicas ($p < 0.05$). La inducción de TNF-α fue 1.5 veces mayor y las células apoptóticas 3-veces mayor en las ratas diabéticas en comparación con las normoglucémicas ($p > 0.05$). El tratamiento con el inhibidor de la caspasa-3 bloqueó significativamente la apoptosis de las células no inflamatorias inducidas por *A. a* en el epitelio gingival y el tejido conectivo ($p > 0.05$). En el estudio donde se colocó la ligadura, la inflamación fue prolongada en las ratas diabéticas y fue revertida con el uso de un inhibidor de TNF, lo que redujo los niveles de mRNA de citoquinas, la infiltración de leucocitos, y el número de osteoclastos. En contraste, la formación de hueso, la formación de osteoide y los números de osteoblastos, aumentaron significativamente frente a los animales diabéticos no tratados. La inhibición de TNF en los animales diabéticos también redujo la apoptosis, aumentó la proliferación de las células que alinean el hueso, y aumentó los niveles de mRNA de FGF-2, TGF β -1, BMP-2, and BMP-6.

Los resultados enlazan la infección de *A. a* con características importantes de destrucción periodontal y ofrece una nueva visión de cómo la diabetes agrava la destrucción periodontal con *A. a* mediante un aumento significativo de la respuesta inflamatoria, lo que lleva al aumento de pérdida ósea y produce un aumento de apoptosis en el epitelio gingival y en las células del tejido conectivo a través de un mecanismo de caspasa-3 dependiente. Los antibióticos tuvieron un efecto más pronunciado en mucho de los parámetros evaluados en las ratas diabéticas que en las normoglucémicas, sugiriendo una deficiencia en la capacidad de los animales diabéticos en combatir la infección. Además la diabetes prolonga la inflamación y la osteoclastogénesis en la periodontitis y a través de TNF limita el proceso normal de reparación modulando negativamente factores que regulan el hueso.

ABSTRACT

A. actinomycetemcomitans que induce la enfermedad periodontal promueve respuestas locales y sistémicas en el periodonto de las ratas.

Objetivo: caracterizar la respuesta histológica y celular de la infección por *A. actinomycetemcomitans (Aa)*.

Material y métodos: ratas Wistar infectadas con *Aa* fueron evaluadas por la respuesta de anticuerpos, la colonización oral por *Aa*, la pérdida de inserción, el reclutamiento de PMN, TNF-α en el epitelio de unión y el tejido conectivo, osteoclastos y la respuesta inmune adaptativa en los ganglios linfáticos locales en el inicio del estudio y en 4, 5 ó 6 semanas después de la infección. A algunos grupos se les dio tratamiento antibacteriano a las 4 semanas.

Resultados: la respuesta de anticuerpos contra *Aa* se produjo dentro de las 4 semanas de la infección, y el 78% de las ratas inoculadas mostraron *Aa* detectable en la cavidad oral ($p < 0,05$). La infección con *Aa* aumentó significativamente la pérdida de inserción que fue revertida por el tratamiento antibacteriano ($p < 0,05$). La expresión de TNF-α en el epitelio de unión siguió el mismo patrón. *Aa* estimuló altamente la formación de osteoclastos y la expresión de TNF-α en el tejido conectivo ($p < 0,05$). El reclutamiento de PMN después de la infección aumentó significativamente ($p < 0,05$). *Aa* también aumentó el número de células T CD8 (+) ($p < 0,05$), pero no las células T CD4 (+) o las células T reguladoras (Treg) ($p > 0,05$).

Conclusión: *Aa* estimuló una respuesta a la infección local que causó un mayor número de PMN, la expresión de TNF-α en el epitelio de unión y la pérdida de inserción. Tanto la expresión de TNF-α en el epitelio de unión como la pérdida de inserción, fue revertido por el tratamiento antibiótico. La infección con *Aa* también aumentó la expresión de TNF-α en el tejido conectivo, los números de los osteoclastos y las células T CD8 (+) en los ganglios linfáticos. La resultados enlazan la infección con *Aa* con importantes características de la destrucción periodontal.

Aggregatibacter actinomycetemcomitans aumenta la apoptosis *in vivo* a través de un mecanismo de la caspasa-3-dependiente en la periodontitis experimental.

El propósito de este estudio fue probar la hipótesis de que la diabetes agrava la destrucción periodontal inducida por la infección de la bacteria *Aggregatibacter actinomycetemcomitans*. Treinta y ocho ratas diabéticas y 33 ratas normales se inocularon con *A. actinomycetemcomitans* y fueron sacrificadas antes de la infección y a las 4, 5, y 6 semanas después de la inoculación. La pérdida de hueso y la infiltración de leucocitos polimorfonucleares (PMN) en el epitelio gingival se midieron en secciones teñidas con hematoxilina-eosina. La inducción del factor de necrosis tumoral alfa (TNF-α) se evaluó por inmunohistoquímica y la expresión de células apoptóticas por la técnica de TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling). Después de la infección con *A. actinomycetemcomitans*, la pérdida de hueso en las ratas diabéticas y la infiltración de PMN fue 1,7 y 1,6 veces mayor respectivamente comparado con las ratas normoglucémicas ($P < 0,05$). La inducción de TNF-α fue 1,5 veces mayor y la de las células apoptóticas fue hasta 3 veces más alta en las ratas diabéticas en comparación con las normoglucémicos ($P < 0,05$). El tratamiento con un inhibidor de la caspasa-3 bloqueó significativamente la apoptosis de células no inflamatorias inducidas por la infección con *A. actinomycetemcomitans* en el epitelio gingival y el tejido conjuntivo ($P < 0,05$). Estos resultados proporcionan una nueva visión de cómo la diabetes agrava la destrucción periodontal inducida por *A. actinomycetemcomitans* en ratas mediante un aumento significativo de la respuesta inflamatoria, lo que lleva a un aumento de la pérdida ósea y mayor apoptosis de las células del epitelio gingival y del tejido conectivo a través de un mecanismo de caspasa-3-dependiente. Los antibióticos tuvieron un efecto más pronunciado en muchos de estos parámetros en ratas diabéticas en comparación con las ratas normoglucémicas, lo que sugiere una deficiencia en la capacidad de los animales diabéticos para resistir una infección.

La diabetes agrava la periodontitis mediante la limitación de la reparación a causa de una mayor respuesta inflamatoria.

La periodontitis es la enfermedad ósea lítica más común y una de las primeras manifestaciones clínicas de la diabetes. La diabetes aumenta el riesgo de periodontitis. El objetivo del presente estudio fue examinar los mecanismos por los cuales la diabetes agrava la periodontitis. La enfermedad periodontal inducida por la colocación de una ligadura se examinó en ratas Goto-Kakizaki con diabetes tipo 2. Un inhibidor del factor de necrosis tumoral (TNF), pegsunercept, se aplicó a las ratas diabéticas después de la aparición de la enfermedad periodontal. Interferon-γ (IFN-γ), TNF-α, interleuquina-1 β (IL-1β), factor de crecimiento de fibroblastos-2 (FGF-2), factor de crecimiento transformante beta-1 (TGFβ -1), la proteína morfogenética ósea 2 (BMP-2), y BMP-6 se midieron a través de la reacción en cadena de la polimerasa con transcriptasa inversa (RT-PCR), y secciones histológicas fueron examinados para medir la infiltración de leucocitos y varios parámetros relacionados con la resorción y formación ósea. La inflamación se prolongó en las ratas diabéticas y se invirtió por el inhibidor de TNF, lo que redujo los niveles de mRNA de citoquinas, infiltración de leucocitos, y los osteoclastos. En contraste, el nuevo hueso y la formación de osteoide y números de osteoblastos aumentó significativamente frente a animales diabéticos no tratados. La inhibición de TNF en animales diabéticos también redujo la apoptosis, aumentó la proliferación de las células que alinean hueso, y aumentó los niveles de mRNA de FGF-2, TGFβ-1, BMP-2 y BMP-6. Por lo tanto, la diabetes prolonga la inflamación y la osteoclastogénesis en la periodontitis y a través de TNF limita el proceso normal de reparación modulando negativamente factores que regulan la formación ósea.

REFERENCES

REFERENCES

1. Pacios S, Violant D, Clotet J. Validation of molecular diagnosis of periodontal pathogens by PCR technique in the Molecular Biology laboratory of the *Universitat Internacional de Catalunya* (UIC). April 2011.

2. Burt B. 2005. Research, Science and Therapy Committee of the American Academy of Periodontology. Position Paper. Epidemiology of Periodontal Diseases. *J Periodontol*. 76: 1406-1419.

3. Boch JA, Wara-aswapati N, Auron PE. 2001. Interleukin 1 signal transduction: current concepts and relevance to periodontitis. J. Dent. Res. 80:400-407.

4. Graves DT, Li J, Cochran DL. 2011. Inflammation and uncoupling as mechanisms of periodontal bone loss. J. Dent. Res. 90: 143-153.

5. Assuma R, Oates T, Cochran D, Amar S, Graves D. 1998. IL-1 and TNF antagonists inhibit the inflammatory responseand bone loss in experimental periodontitis. J Immun. 160: 403-409.

6. Delima A, Spytos K, Amar S, Graves DT. 2002. Inflammation and tisue loss caused by periodontal pathogens is reduced by IL-1 antagonists. J Infect Dis. 186: 511-516.

7. Teng Y, Nguyen H, Gao X, et al. 2000. Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. J Clin Invest. 106: R59-67.

8. Han X, Kawai T, Eastcott JW, Taubman MA. 2006. Bacterial-responsive B lymphocytes induce periodontal bone resorption. J Immunol. 176: 625-631.

9. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. 1998. Microbial complexes in subgingival plaque. J Clin Periodontol. 25(2): 134-44.

10. Van Winkelhoff AJ, Loos BG, van der Reijden WA, van der Velden U. 2002. Porphyromonas gingivalis, Bacteroides forsythus and other putative periodontal pathogens in subjects with and without periodontal destruction. J. Clin. Periodontol. 29: 1023–1028.

11. American Academy of Periodontology. 2000. Parameter on aggressive peri-odontitis. J. Periodontol. 71(5 Suppl): 867–9.

12. Slots J, Reynolds HS, Genco RJ. 1980. Actinobacillus actinomycetemcomitans in human periodontal disease: a cross-sectional microbiological Investigation. Infect.

Immun. 29(3): 1013–20.

13. Lang, N.P., Bartold, P.M., Cullinan, M., Jeffcoat, M., Mombelli, A. et al. 1999. Consensus Report: Aggressive Periodontitis. Ann Periodontol. 4: 53.

14. Loe H, Brown LJ. 1991. Early onset periodontitis in the United States of America. *J Periodontol*. 62: 608-616.

15. Armitage GC, Cullinan MP, Seymour GJ. 2010. Comparative biology of chronic and aggressive periodontitis: introduction. Periodontol 2000. 53: 7-11.

16. Mandell RL, Socransky SS. 1998. Microbiological and clinical effects of surgery plus doxycycline on juvenile periodontitis. J. Periodontol. 59: 373-379.

17. Christersson LA. 1993. *Actinobacillus actinomycetemcomitans* and localized juvenile periodontitis: clinical, microbiologic, and histologic studies. Swed. Dent. J. Suppl. 90: 1-46.

18. Fine DH, et al 2007. Aggregatibacter actinomycetemcomitans and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. J. Clin. Microbiol. 45: 3859-3869.

19. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. 1999. Virulence factors of *Actinobacillus actinomycetemcomitans. Periodontol 2000*. 20:136-167.

20. Kinane DF, Hart TC. 2003. Genes and gene polymorphisms associated with periodontal disease. Crit Rev Oral Biol Med. 14(6): 430-49.

21. Henderson B, Nair S, Ward J, Wilson M. 2003. Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. 57: 29-55.

22. Brown SA, Whiteley M. 2007. A novel exclusion mechanism for carbon resource partitioning in Aggregatibacter actinomycetemcomitans. J Bacteriol. 189(17): 6407-14.

23. Müller HP, Heinecke A, Fuhrmann A, Eger T, Zöller L. 2001. Intraoral distribution of Actinobacillus actinomycetemcomitans in young adults with minimal periodontal disease. J Periodontal Res. 36(2): 114-23.

24. Guthmiller JM, Lally ET, Korostoff J. 2001. Beyond the specific plaque hypothesis: are highly leukotoxic strains of Actinobacillus actinomycetemcomitans a paradigm for periodontal pathogenesis? Crit Rev Oral Biol Med. 12(2): 116-24.

25. Schreiner HC, Sinatra D, Kaplan JB, et al. 2003. Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proc Natl Acad Sci USA*.100: 7295-7300.

26. Paju S, Carlson P, Jousimies-Somer H, Asikainen S. 2000. Heterogeneity of Actinobacillus actinomycetemcomitans strains in various human infections and relationships between serotype, genotype, and antimicrobial susceptibility. J Clin Microbiol. 38(1): 79-84.

27. Paju, S., Carlson, P., Jousimies-Somer, H. & Asikainen, S. 2003. Actinobacillus actinomycetemcomitans and Haemophilus aphrophilus in systemic and nonoral infections in Finland. APMIS 111, 653–657.

28. Rahamat-Langendoen JC, van Vonderen MG, Engström LJ, Manson WL, van Winkelhoff AJ, Mooi-Kokenberg EA. 2011. Brain abscess associated with Aggregatibacter actinomycetemcomitans: case report and review of literature. J Clin Periodontol. 38(8): 702-6.

29. Paturel, L., Casalta, J. P., Habib, G., Nezri, M. & Raoult, D. 2004. Actinobacillus actinomycetemcomitans endocarditis. Clin Microbiol Infect. 10, 98–118

30. Henderson B, Wilson M, Sharp L, Ward JM. 2002. Actinobacillus actinomycetemcomitans. J Med Microbiol. 51(12): 1013-20. Review.

31. Kelk P, Abd H, Claesson R, Sandström G, Sjöstedt A, Johansson A. 2011. Cellular and molecular response of human macrophages exposed to Aggregatibacter actinomycetemcomitans leukotoxin. Cell Death Dis. 2: e126.

32. Haubek D, Ennibi OK, Poulsen K, Vaeth M, Poulsen S, Kilian M. 2008. Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* in Morocco: a prospective longitudinal cohort study. *Lancet*. 371: 237-42.

33. Schreiner, H., Markowitz, K., Miryalkar, M., Moore, D., Diehl, S. & Fine, D. H. 2011. *Aggregatibacter actinomycetemcomitans*-induced bone loss and antibody response in three rat strains. *Journal of Periodontology.* 82, 142-150.

34. Graves DT, Kang J, Andriankaja O, Wada K, Rossa C Jr. 2012. Animal models to study host-bacteria interactions involved in periodontitis. Front Oral Biol. Review. 15: 117-32.

35. Graves D.T., Daniel Fine, Yen-Tung A. Teng, Tom E. Van Dyke, and George Hajishengallis. 2008. The Use of Rodent Models to Investigate Host-Bacteria Interactions Related to Periodontal Disease. *J Clin Periodontol*. 35(2): 89-105.

36. Makhoul H, Bashutski J, Halubai S, Dabiri D, Benavides E, Kapila YL. 2013. Apoptotic activity of gingival crevicular fluid from localized aggressive periodontitis. J Int Acad Periodontol. 15(1): 2-7.

37. Schreiner HC, Sinatra K, Kaplan JB, Furgang D, Kachlany SC, Planet PJ, Perez BA, Figurski DH, Fine DH. 2003. Tight-adherence genes of Actinobacillus actinomycetemcomitans are required for virulence in a rat model. Proc Natl Acad Sci U S A. 100: 7295-7300

38. Schreiner H, Markowitz K, Miryalkar M, Moore D, Diehl S, Fine DH. 2011. Aggregatibacter actinomycetemcomitans-induced bone loss and antibody response in three rat strains. J Periodontol. 82: 142-150

39. Li Y, Messina C, Bendaoud M, Fine DH, Schreiner H, Tsiagbe VK. 2010. Adaptive immune response in osteoclastic bone resorption induced by orally administered Aggregatibacter actinomycetemcomitans in a rat model of periodontal disease. Mol Oral Microbiol. 25: 275-292

40. Smith PC, Guerrero J, Tobar N, Cáceres M, González MJ, Martínez J. 2009. Tumor necrosis factor-alpha-stimulated membrane type 1-matrix metalloproteinase production is modulated by epidermal growth factor receptor signaling in human gingival fibroblasts. J Periodontal Res. 44(1): 73-80.

41. Sigusch, B. W., Wutzler, A., Nietzsch, T. & Glockmann, E. 2006. Evidence for a specific crevicular lymphocyte profile in aggressive periodontitis. *Journal of Periodontal Research.* 41, 391-396.

42. Artese, L., Simon, M.J., Piattelli, A., Ferrari, D.S., Cardoso, L.A., Faveri, M., Onuma, T., Piccirilli, M., Perrotti, V. & Shibli, J.A. 2011. Immunohistochemical analysis of inflammatory infiltrate in aggressive and chronic periodontitis: a comparative study. *Clinical Oral Investigation.* 15, 233-240.

43. Lima, P.M., Souza, P.E., Costa, J.E., Gomez, R.S., Gollob, K.J. & Dutra W.O. 2011. Aggressive and chronic periodontitis correlate with distinct cellular sources of key immunoregulatory cytokines. *Journal of Periodontology.* 82, 86-95.

44. Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE. 2011. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 157: 1851–1875.

45. Ohara M, Miyauchi M, Tsuruda K, Takata T, Sugai M. 2011. Topical application of *Aggregatibacter actinomycetemcomitans* cytolethal distend- ing toxin induces cell cycle arrest in the rat gingival epithelium in vivo. J. Periodontal Res. 46: 389 – 395.

46. Heller D, et al. 2011. Impact of systemic antimicrobials combined with anti- infective mechanical debridement on the microbiota of generalized ag- gressive periodontitis: a 6-month RCT. J. Clin. Periodontol. 38: 355–364.

47. Xajigeorgiou C, Sakellari D, Slini T, Baka A, Konstantinidis A. 2006. Clinical and microbiological effects of different antimicrobials on generalized aggressive periodontitis. J. Clin. Periodontol. 33: 254 –264.

48. Yek EC, et al. 2010. Efficacy of amoxicillin and metronidazole combina- tion for the management of generalized aggressive periodontitis. J. Periodontol. 81: 964 – 974.

APPENDIX

APPENDIX

Appendix 1. Species and number of Animals

Species

Male Goto-Kakizaki (GK) rat and male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Taking into account that approximately 20% of the GK rats would not became diabetic it was purchased 20% rats of excess. GK rat is a substrain from Wistar rats, which were used as a non-diabetic control. Gk are non-obese rats that develop Type 2 diabetes mellitus around the age of 12 weeks. They develop diabetes spontaneously and it is thought to imply more than 1 gene.

Animals were provided to the University of Pennsylvania (UPenn) at the age of 8 weeks. To control once they became diabetics, blood glucose was obtained from a tail Nick. It was performed once weekly and measured with the use of a digital glucometer. Once glucose levels reached 220mg/dl they were considered as diabetic. GK rats that did not showed hyperglycemia (serum glucose levels >220mg/dl) by 16 weeks of age were euthanized.

Total number of animals:

Rat, Goto-Kakizaki on Wistar background. Number: 173.

Rat, Wistar. Number: 120.

Species justification:

The rats periodontal anatomy, histopathology of periodontal lesion, and basic immunobiology resemblance to human. The Goto-Kakizaki (GK) rat is a non-obese Wistar substrain which develops Type 2 diabetes mellitus early in life. As a well-known type 2 diabetes animal model, GK rat is an important animal model to our study to prove that type 2 diabetes increased cell death which may contribute to the periodontal bone loss. Since GK rat were developed from Wistar rat, we used Wistar rat as the experiment control animal.

Animal numbers:

To calculate the sample size a power analysis was undertaken. A type I error frequency was set at 5% (a significance level, alpha, of 0.05) and the power of the statistical test was set at 90% (P=0.9, beta=01). Based on Preliminary Data and previous experiments with cytokine antagonists, it was anticipated that a change of around 50% would represent a significant difference between control and experimental group. On this basis a sample size of 8 per group provided a sufficient number of specimens to achieve statistical significance for histologic analysis.

Total experimental animals for set 1 experiment: 32+72= 104 (Appendix 5)

Group 1: examine the different degree of periodontal bone loss between GK and WK on animal model of ligature-induced periodontitis.

Strains: 2 (GK and WK)

Time points: 2 (0 day and 7 day)

Group size (N): 8

Subtotal: 8*2*2=32

Group 2: test the effect of TNF blocker.

Strains: 2 (GK and WK)

Time points: 3 (11 day, 15 day and 22 day)

Treatment group: 2 in the GK (Z-DEVD-FMK and DMSO vehicle)

Group size (N): 8

Subtotal: 8*3*3=72

	Group 1		Group 2		
Condition	Normal	Diabetic	Normal	Diabetic	Diabetic
Euthanize	0 and 7 days	0 and 7 days	11, 15 and 22 days	11, 15 and 22 days	11, 15 and 22 days
Administration product					TNF- α inhibitor (Pegsunrecept)

Table 1. A ligature was placed in all the time points except on baseline. Animals were sacrificed at the moment of the ligature removal (day 7), 4 (day 11), 8 (day 15) and 15 (day 22) days after.

Total experimental animals for set 2 experiment: 64+80+16= 160 (Appendix 4)

Group 1: examine changes caused by diabetes by comparing GK diabetic and Wistar normoglycemic rats.

Number of strains: 2 (GK and WK)

Time points: 5 (0, week 4, week 5 and week 6)

Group size (N): 8

Subtotal: 8*4*2=64

Group 2 and 3a and b: test the effect of antibiotic treatment and the effect of caspase-3 inhibitor. Starting at 4 weeks (both GK and Wistar rats were received antibiotics and were compared to rats in group 1)

Number of strains: 2 (GK and WK)

Time points: 2 (week 5 and week 6)

Treatment group: 2

Group size (N): 8

Subtotal: 8*2*2*2+16=80

Group 3C: test the vehicle alone to GK rat.

Number of strains: 1

Time points: 2 (5wk and 6wk)

Group size (N): 8

Subtotal: 8*2*1=16

	Group 1 (*A.a*)		Group 2 (*A.a*+AB started at week 4)		Group 3 (*A.a*+AB started at week 4)		
Condition	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Diabetic (c)
Euthanize	0, 4, 5 and 6 weeks	0, 4, 5 and 6 weeks	5 and 6 weeks	5 and 6 weeks	5 and 6 weeks	5 and 6 weeks	5 and 6 weeks
Administration						Caspase 3-inh	Vehicle (DMSO/PBS)

Table 2. A total of 4 different time points were used in the study. Some of the animals were treated with a second antibiotic treatment and with a caspase-3 inhibitor

In conclusion, the total experimental animals were 264. From these animals, 104 belong to the experimental set 1 and 160 belong to the experimental set 2. From these 264 animals, 144 animals were GK rat. Considering that 20% of Gk rat would not be becoming diabetic, actual number of GK animals needed was 144*20%+144= 173 animals.

The experiment was accomplished by 2 sets of experiments. In set 1 periodontal disease was initiated by tying a ligature around the maxillary molar teeth and in Set 2 periodontal disease was initiated by the inoculation of *A.actinomycetemcomitans* bacteria into the oral cavity.

Appendix 2. Hazardous materials used

Different substances were used during the experiments.

To minimize direct contact, exam gloves were worn that overlap the sleeve of the laboratory coat, laboratory personal thoroughly washed hands after use, safety goggles and laboratory coats were worn.

Substance	Dose	Route of Administration	Poses hazard to humans through direct or indirect contact with the animal and/or its bedding
Chlorexidine gluconate 0.12% (periogard also contains saccharine)	Approx. 0.3 ml of 0.12% solution swabbed on teeth and oral mucosa	Swabbed on teeth and oral mucosa. Swab mouth with cotton. Tipped applicator.	No
Kanamycin, ampicillin	20 mg/day	Ad libitum in drinking water	No
1-3% Isoflurane	1-2 minute	Inhalant	No
Alizarin	30 mg/kg	IP	No
Z-DEVD-FMK (peptide)	2 mg/kg	IP	No
Pegsunercept	4 mg/kg	IP	No
Calcein	10 mg/kg	IP	No

Appendix 3. Animal identification, blood collection and fluorescent dyes

Animal identification:

Animals were identificated by ear punching: right ear only, left ear only, or both side ears.

Blood collection

Isoflurane was administred. The tail was swabbed with an alcohol wipe. Blood was drawn from a lateral tail vein with a needle that is smaller than 23-gauge needle prior to antibiotic pre-treatment at the start of the experiments and and again at time of the euthanasia. Approximately 100 ul was drawn. Weekly collections of blood at other time points was obtained by small tail laceration (around 20 ul) after swabbing with alcohol. Mild pressure with sterile gauze was used to stop bleeding if iccured

Species: rat
Anatomic site: tail vein
Volume collected: <25 ul; 100 ul (ml/kg)
Bleeding interval/frequency: once weekly <25 ul each time; two times 100 ul each time with more that 3 weeks of interval.

Fluorescent dyes.

Injection of fluorescent dyes was performed by i.p. injection at 2 time points. No anesthesia was required.

Appendix 4. Experimental Set 2

Blood glucose levels were assessed for GK and Wistar rats as above. Once GK rats became diabetic they and age matched Wistar rats were prepared for bacterial inoculation by antibiotic pre-treatment. This was done to facilitate colonization by inoculated bacteria.

Pre-antibiotic treatment

Pre-treatment consisted of Kanamycin and ampicillin (20mg of each antibiotic daily per rat) daily in the drinking water for four days. For the last two days of antibiotic treatment the rats' mouths were swabbed with a clorhexidine gluconate 0.12% rinse. Because the rats physiologically chewed on the cotton swab and liked the taste of clorhexidine (which has been sweetened with saccharin for human use), the anesthesia of the animals was not performed.

Aa inoculation

After 3 days of the antibiotic treatment, *A. actinomycetemcomitans* (10^9CFU in 25-50 ul of steile PBS) was administered by applying a pipette to the oral cavity daily for 4 days. At the same time the bacteria was applied in their feed. The inoculation by feeding helps in colonization of the oral cavity by the exogenous bacteria and was carried out as follows. They regular food was removed at 9 am in the morning for 3 hours. After that, 1 gram of powdered food mixed with bacteria (10^9CFU) was given in feeder trays so that it was controlled that the same amount of *A. actinomycetemcomitans* was eaten per each animal.

After the dose per animal was consumed in a given day, rats were placed on powdered food without *A. actinomycetemcomitans* for the rest of the day ad libitum. For 2 weeks during 5 days this procedure was repeated. The end of the feeding regimen represented the start of the experiment (Day 0).

Sampling of oral cavity microflora

Sampling of oral bacteria was taken at different time points: prior to antibiotic pre-treatment, at day 0, at 5 weeks after the inoculation of the bacteria and at the time of the euthanasia. This was done under anesthesia by swab with sterile cotton tipped applicator for the soft tissue and with a sterile toothpick for the tooth surface.

Termination of the infection

To study the resolution of periodontal disease, the infection was stopped by treating the rats with antibiotic. They were treated with antibiotics (the same used for pre-treatment) at the 4-week time point during four days daily. They also received at the same time a daily rinse with chlorhexidine gluconate (0.12%).

Inhibition of caspase-3:

Caspase-3 blocker ZDEVD-FMK, was given daily for two weeks starting at week 4 by intraperitoneal injection.

Fuorescent dyes

To measure the amount of bone formed, the animals were injected intraperitonealy with the fluorescent dyes calcein (10mg/kg) and alizarin complexone (30mg/kg) 1 day before euthanasia. The sequential administration of fluorescent dyes allowed us to measure the amount of mineralized bone formation. Bone formation was measured under a fluorescent microscope as the area of the 2 lines formed by the dyes deposited.

Euthanasia

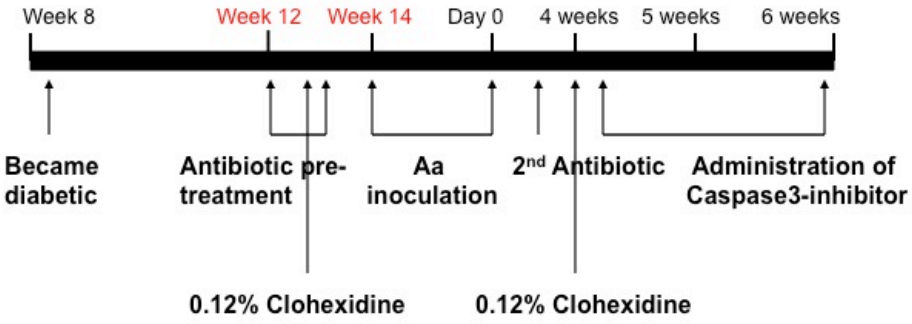
8 different groups of around 7 animals each were used for the study. A group of animals (baseline) were euthanized previous of the *Aa* infection. 4, 5 and 6 week after the rest the rest of groups were euthanized. On 5 and 6 weeks the two diabetic groups were treated with AB and with AB+inhibitor.

Processing of the specimens

After euthanasia the head was surgically removed, the maxilla with molar teeth collected and prepared for sectioning by fixation in paraformaldehyde. Rat lymph node cells were prepared and analyzed by flow cytometry. For hematologic analysis 1 ml of blood was collected from each rat at time of euthanasia into tubes with EDTA.

Methods:

Experimental design



Appendix 5. Experimental Set 1

After GK rats became diabetic 3 weeks were elapsed before ligatures were placed. Rats were around 15 weeks old. The ligatures were placed around the 1st and 2nd maxillary molars to induce periodontal disease. Blood glucose levels were assessed once weekly and rats were weighed once weekly when placed the ligatures.

Ligature placement

GK diabetic and age matched control Wistar rats were anesthetized by intraperitoneal injection of Ketamine/xylazine. Ligatures (4 "0" silk surgical sutures or equivalent size cottom thread) were placed around 1st and 2nd molars. The silk ligature was positioned around the neck of the tooth and tying it so that it surrounds the tooth at the gingival margin but does not constrict the gingiva. Ligatures were tied using standard surgical square knots. For ligature placement the mouth was slightly kept open with small tissue forceps. The ligature causes the accumulation of plaque and it creates the perfect environment to induce periodontal disease. After placement of ligatures animals were monitored once daily for signs of stress though very few difficulties are usually reported in rats placement of ligatures. Rats were anesthetized during ligature placement with either ketamine/xylazine (or isoflurane).

TNF- α inhibitor

Diabetic rats received injection of TNF-α inhibitor (pegsunercept) provided by Amgen (Thousand Oaks, CA, USA) or its vehicle (PBS) at the time of the ligature removals and every 3 days thereafter. The number of injections of the TNF inhibitor or matched vehicle alone (PBS) was 5 times (applied on day 7, 10, 13, 16, and 19).

Euthanasia

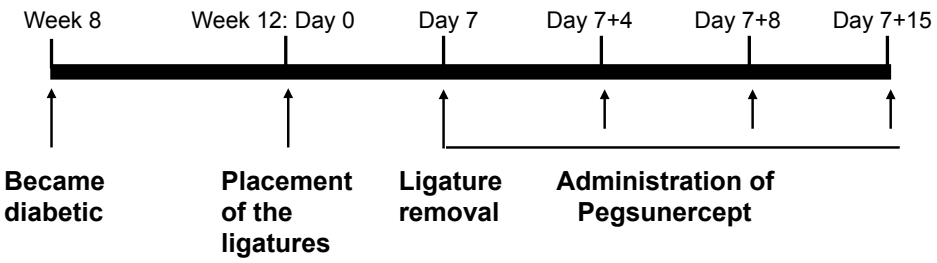
13 different groups of around 7 animals each were used for the study. A group of animals (baseline) were euthanized previous of the ligature. A second group was euthanized on day 7 when ligatures were removed. Ligatures were removed under anesthesia (typically ketamine/xylazine but occasionally isoflurane). The remaining diabetic and normoglycemic rats were treated with a TNF inhibitor or vehicle for 11, 15 and 22 days to examine events after periodontal disease was initiated.

Processing of the specimens

Immediately after euthanasia cardiac puncture was performed to obtain blood and do the measurement of glycosylated hemoglobin. After euthanasia the head was surgically removed, the maxilla with molar teeth collected and prepared for sectioning by fixation in paraformaldehyde or stored in liquid nitrogen for RNA or protein extraction.

Methods:

Experimental design



Appendix 6. Presentations

- SDM Retreat. May 31, 2013. Philadelphia, PA United States. **Pacios S**, Shah H, Mattos M, Xiao W, Graves DT. Abstract submitted Entitled: *Osteoblasts lineage cells play a central role in bacteria induced periodontitis.*
- SDM Retreat. May 31, 2013. Philadelphia, PA United States. Xiao W, Azizyan D, **Pacios S**, Alnammary M, Graves DT. Abstract submitted Entitled: *FOXO1 Deletion in Dendritic Cells Leads to Bacteria Induced Osteoclastogenesis and Periodontitis.*
- SDM Retreat. May 31, 2013. Philadelphia, PA United States. Dong G, Thatikonda SA, Cramer C, Sun AH, Wang Y, Xiao W, **Pacios S**, Xu F, Tian C, Graves DT. Abstract submitted Entitled: *FOXO1 expression in dendritic cells is needed to activate the adaptive immune response.*
- SDM Retreat. June 8, 2012. Philadelphia, PA United States. **Pacios S**, Kang J, Galicia J, Gluck Kenneth, Patel H, Ovaydi A, Petrov S, Alawi F, Graves D. Poster Entitled: *Cellular mechanisms that affect bone formation during the resolution of inflammation in periodontitis in diabetes.*
- SDM Retreat. June 8, 2012. Philadelphia, PA United States. Xiao W, **Pacios S**, Graves DT. Poster Entitled: *The effect of inhibition NFkB specifically in osteoblasts on bone formation in periodontitis mice model.*
- EuroPerio 7, 7º Congress of the European Federation of Periodontology. 6-9 June, 2012. Vienna, Austria. Kang J, Bezerra BB, Andriankaja O, **Pacios S**, Bae H, Schriner H, Fine D, Graves DT. Poster Entitled: *Diabetes enhances inflammatory response and apoptosis of periodontium in Aggregatibacter actinomycetemcomitans infected rats.*
- Penn Center for Musculoskeletal Disorders Annual Scientific Symposium. Wednesday, October 26, 2011. Philadelphia, PA United States. **Pacios S**, Kang J, Galicia J, Gluck Kenneth, Patel H, Ovaydi A, Petrov S, Alawi F, Graves DT. Poster Entitled: *Bone formation in inflammatory conditions.*
- Gordon Research Conference on Periodontal Diseases. July 17, 2011 - July 22, 2011 at Davidson College in Davidson, NC United States. **Pacios S**, Kang J, Galicia J, Gluck Kenneth, Patel H, Ovaydi A, Petrov S, Alawi F, Graves DT. Poster Entitled: *TNF-a affects bone formation during the resolution of the*

inflammation in diabetes ligature-induced model.

- Gordon Research Conference on Periodontal Diseases. July 17, 2011 - July 22, 2011 at Davidson College in Davidson, NC United States. Kang J, Bezerra B, Andriankaja O, **Pacios S**, Bae Hyung, Schriner H, Fine D, Graves DT. Poster Entitled: *Diabetes aggravates periodontal destruction in Aggregatibacter actinomycetemcomitans infected rats.*