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Development of an Animal Model for *Aggregatibacter Actinomycetemcomitans* Biofilm-Mediated Oral Osteolytic Infection: A Preliminary Study

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Abstract

Background—Biofilm-induced inflammatory osteolytic oral infections, such as periodontitis and peri-implantitis, have complex etiology and pathogenesis. A significant obstacle to research has been the lack of appropriate animal models where the inflammatory response to biofilms can be investigated. The aim of this study is to develop a novel animal model to study the host response to *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*)—biofilm colonizing titanium implants.

Methods—Titanium implants were inoculated in vitro with *A. actinomycetemcomitans*, establishing a biofilm for 1 to 3 days. Biofilm-inoculated and control implants were transmucosally placed into rat hard palate or alveolar ridge. Analysis included documentation of clinical inflammation, polymerase chain reaction, and culture detection of *A. actinomycetemcomitans* and microcomputed tomography quantitation of peri-implant bone volume.

Results—Viable *A. actinomycetemcomitans* biofilm was successfully established on titanium implants in vitro, detected by confocal laser scanning microscopy. An inflammatory response characterized by clinical inflammation, bleeding, ulceration, hyperplasia, and necrosis was observed around biofilm-inoculated implants. *A. actinomycetemcomitans* was detected by polymerase chain reaction and culture analysis on 100% of biofilm-inoculated implants for up to 3 weeks and 25% for up to 6 weeks. Microcomputed tomography analysis demonstrated significantly

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lower bone volume ($P < 0.05$) around biofilm-inoculated implants ($29.6\% \pm 7.6\%$) compared to non-inoculated implants ($50.5\% \pm 9.6\%$) after 6 weeks.

Conclusions—These results describe a novel animal model where *A. actinomycetemcomitans* biofilm was established in vitro on titanium implants before placement in rat oral cavity, leading to an inflammatory response, osteolysis, and tissue destruction. This model may have potential use for investigation of host responses to biofilm pathogens and antibiofilm therapy.

Keywords

Aggregatibacter actinomycetemcomitans; biofilms; dental implants; infection; models; animal; rats

Periodontitis represents one of the most common infections in humans and refers to an inflammatory destruction of the periodontium (i.e., tooth-supporting structures). Likewise, in the related periimplantitis destructive inflammatory changes take place in the tissues surrounding dental implants, representing the most common inflammatory complication in orofacial implantology.¹ The clinical and histopathologic features of periodontitis and peri-implantitis are similar including erythema, pocket formation, mobility, bleeding on probing, connective tissue changes, inflammatory infiltrate, and osteolytic bone loss.^{1–6}

Despite research into periodontitis and peri-implantitis, the exact mechanisms involved have not been elucidated. Available evidence suggests that pathogenic organisms that inhabit oral biofilms are important triggers in both diseases, whereas much of the tissue destruction is the result of adaptive and innate host-mediated immune responses to biofilm pathogens.^{1,7,8} Biofilm organisms differ considerably from their planktonic counterparts because they are characterized by cells that are not simply attached to a surface, but have developed into a community.⁹ They are embedded in a matrix of extracellular polymeric substances that the cells have produced to connect and communicate with each other. Furthermore, biofilm organisms exhibit an altered phenotype with respect to growth rate, gene transcription, and antimicrobial resistance.^{10–12}

An important obstacle preventing advancement in the field has been the lack of a well-controlled animal model to investigate the interactions between the biofilm organisms and the immune response. Current animal models for periodontitis involve inoculation of specific pathogenic bacteria through oral gavage or injection into oral tissues.¹³ To increase bacterial retention, ligatures are commonly placed circumferential to teeth or implants.^{14–17} The problems with these current models include: 1) bacteria are applied in a planktonic form and at a growth phase inconsistent with their biofilm form;¹⁸ 2) bacteria must compete with the indigenous flora in an oral biofilm, and consequently, these bacteria rarely persist to a point where viable organisms can be recovered from the animals; 3) introduced bacteria are presented to the host as an acute antigenic challenge, which may or may not mimic the pathogenic process; and 4) ligature-induced tissue destruction does not accurately recapitulate in vivo pathogenesis. The complex nature of multispecies biofilms that are associated with both conditions at disease sites does not allow for accurate characterization to determine which organism is functioning as a pathogen by activating the local immune response.¹⁹ A more valid model system requires that a specific etiologic biofilm pathogen be successfully implanted and recovered at will, to facilitate studying the genotypic and phenotypic effects of the disease state on both the host and the pathogen.

It was hypothesized that *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) can form a biofilm on titanium implants, which can in turn be used as a colonizing surface, allowing in vivo bacterial persistence and inflammatory host response. To that end, the aim of this study is to develop a novel animal

model to study the host response to biofilm colonizing titanium implants. *A. actinomycetemcomitans* was chosen in this study for the following reasons: 1) the availability of many research tools in our group, such as a collection of *A. actinomycetemcomitans* strains from different clinical conditions (e.g., periodontitis, non-periodontitis oral sites, extraoral infections, and a number of specific gene deletion mutants); 2) *A. actinomycetemcomitans* is an important oral pathogen that has been shown to be associated with both periodontitis and peri-implantitis;^{20–22} and 3) *A. actinomycetemcomitans* readily forms biofilms in vitro, which could be potentially transferred to animals. Our results demonstrate for the first time that titanium oral implants can be used as a colonizing surface for *A. actinomycetemcomitans* biofilms and when implanted transmucosally in the rat jaw, *A. actinomycetemcomitans* biofilm successfully persisted. Moreover, a clinical inflammatory response and osteolysis and tissue destruction resulted from the placement of biofilm-colonized titanium implants.

MATERIALS AND METHODS

Bacteria and Culture Conditions

A. actinomycetemcomitans strain D7S-1 was originally recovered from a patient with aggressive periodontitis.^{23,24} *A. actinomycetemcomitans* strain D7S-1 (serotype a) was grown on modified trypticase soy broth (mTSB; 3% trypticase soy broth with 0.6% yeast extract) for 2 to 3 days before five to 10 colonies were transferred to 5 mL of liquid mTSB, vortexed to disperse the bacteria, and the suspension transferred to 1 to 2 mL of fresh mTSB at a 1:20 dilution and incubated at 37°C with 5% CO₂.

Implant Surface Treatment and Biofilm Cultivation

The surface of machined 1.2 × 4.5 mm titanium implants[#] was modified by grit blasting with Al₂O₃ (100 μm) and HCl etching (pH 3, 20 minutes, 80°C) to produce rough surfaces in addition to the machined-implant surfaces before *A. actinomycetemcomitans* inoculation. The superficial roughness of an implant is thought to favor bacterial adhesion and colonization when exposed to the oral environment in vivo.²⁵ The machined and rough implants were completely submerged in mTSB inoculated with *A. actinomycetemcomitans* D7S-1 for 1 to 3 days at 37°C with 5% CO₂.

Bacterial Viability

Viability of *A. actinomycetemcomitans* biofilm cells was evaluated after 3 days of culture on the implant surfaces by live/dead staining.²⁶ A fluorescing cell system^{**} was used for direct visualization and analysis of the *A. actinomycetemcomitans* biofilm. The system is composed of two nucleic acid-binding stains: SYTO 9, which penetrates all bacterial membranes (stains the cells green); and propidium iodide, which only penetrates cells with damaged membranes (stains the cells red). The combination of the two stains produces yellow fluorescing cells. Optimal incubation conditions for visualization were found to be 15 to 20 minutes at room temperature in the dark. Total (red plus green) and viable (green) cells were visualized with confocal laser scanning microscopy.

Animals

Thirty 5-month-old, virgin, female rats^{††} were housed in a laboratory at 22.2°C under a 12-hours light and 12-hours dark cycle and fed *ad libidum*.^{‡‡} All animals were treated

[#]Ace Surgical Supply, Brockton, MA.

^{**}BacLight Molecular Probes, Invitrogen, Carlsbad, CA.

^{††}Sprague-Dawley, Charles River Laboratories, Hollister, CA.

^{‡‡}Purina Laboratory Rodent Chow, Purina Milk, Richmond, IN.

according to the guidelines and regulations for the use and care of animals at the University of Southern California. To investigate the suitability of hard palate as the recipient site, one implant was transmucosally placed into the hard palate in the midline area in the molar region. Accordingly, implants were placed into 24 rats (six control machined, six control rough, six *A. actinomycetemcomitans* biofilm–inoculated machined, and six *A. actinomycetemcomitans* biofilm–inoculated rough) and followed for 1 to 14 days. To investigate the suitability of alveolar ridge as the recipient site, one implant was transmucosally placed on each side into alveolar ridge natural diastema that exists between maxillary molars and incisors (total of two implants per animal) of six rats and followed for 1 to 6 weeks. For each set of experiments, half of the implants were inoculated with *A. actinomycetemcomitans* biofilm and the other half were control sham-inoculated implants. In the alveolar ridge study, both implants were either inoculated with *A. actinomycetemcomitans* biofilm or were control implants. At the end of the treatment period, the rats were euthanized and the skulls were harvested and stored in 10% buffered formalin for further analysis.

Scanning Electron Microscopy

Titanium implants inoculated with *A. actinomycetemcomitans* and controls after 1 to 3 days in vitro were visualized by scanning electron microscopy. Implant samples were prepared with Karnovsky fixative (6% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer) for 2 hours. After two washes in 0.1M cacodylate buffer for 15 minutes, the specimens were post-fixed with 2% osmium tetroxide in 0.2M cacodylate buffer for 2 hours at 4°C and dehydrated with ascending ethanol concentration (30% to 70%). After an increasing concentration gradient of hexamethyldisilazane, specimens were embedded in 100% hexamethyldisilazane at room temperature for 24 hours and air-dried. After gold coating, the preparations were observed with JSM-6610 scanning electron microscope at 30 kV.

Polymerase Chain Reaction and Culture Detection of an *A. actinomycetemcomitans* Biofilm on the Surgical Implants

To confirm the presence of *A. actinomycetemcomitans* biofilms on the implants, bacterial samples of control and *A. actinomycetemcomitans* biofilm implants were collected from the oral surfaces of implants after 1 to 14 days and after 1 to 6 weeks. Samples were harvested by touching a sterile tooth-pick on surgically placed implants for 5 to 10 seconds, followed by vortexing in 500 µL of phosphate-buffered saline. *A. actinomycetemcomitans* was detected by both polymerase chain reaction (PCR) and culture identification methods. The PCR was based on amplification of 16S ribosomal RNA gene (16S RNA) signature genes (primer sequence for species-identity PCR forward: 5' AAA CCC ATC TCT GAG TTC TTC TTC 3' and reverse 5' ATG CCA ACT TGA CGT TAA AT 3') using standard conditions for *Taq* DNA polymerase.²⁵ As a positive control, the 16S rDNA was amplified from *A. actinomycetemcomitans* D7S-1 DNA template, and for a negative control, no template was added to the reaction. For culture detection, a 1:10 dilution was made in mTSB and was plated in triplicate on trypticase-soy with serum, bacitracin, and vancomycin. After incubation at 37°C with 5% CO₂ condition for 3 to 5 days, *A. actinomycetemcomitans* colonies on trypticase-soy with serum, bacitracin, and vancomycin plates were identified under a stereomicroscope and counted to postulate the density of *A. actinomycetemcomitans* on the implant surface. The detection of *A. actinomycetemcomitans* at 1:10 dilution was considered positive.

Microcomputed Tomography

Microcomputed tomography (µCT) radiologic evaluation with concomitant three-dimensional reconstruction of peri-implant bone and tissues was performed after 14 days for

the center of the palate and at 6 weeks after placement only for alveolar ridge regions. Each rat was placed in a sample holder in the cranial-caudal direction and scanned using a high-resolution μ CT system^{§§} at a spatial resolution of 18.676 μ m (voxel dimension) and 1,536 $\times$ 1,536 pixel matrices. Rats were maintained under general anesthesia with isoflurane during the scanning procedure. After scanning, the two-dimensional image data were stored in the Digital Imaging and Communications in Medicine (DICOM) format, transferred to a computer, and a three-dimensional reconstruction and analysis was performed. To reduce the size of data for computation, the craniofacial region was cropped and saved from the obtained consecutive microtomographic slice images as a volume of interest using software.^{|||} In this step, the original spatial resolution was maintained, because data were not resampled. The peri-implant bone volume was measured using software.^{¶¶} The bone tissue was segmented using a global thresholding procedure. Threshold equaling -360 HU was used to investigate peri-implant bone tissues. The peri-implant bone was measured by applying a virtual cylindrical divider, whose diameter was 50 voxels in diameter and 105 voxels in length. The proportion of bone volume occupying this virtual cylindrical space around implants was measured and compared. The Student *t* test was used for pairwise comparisons of biofilm-inoculated implants bone volume (*n* = 3) compared to controls (*n* = 3). Statistical significance was given at *P* < 0.05.

RESULTS

Surface Characteristics of Titanium Implants

To increase the surface area and roughness of titanium implants to promote establishment of *A. actinomycetemcomitans* biofilms, the surfaces of machined-titanium implants were modified by grit blasting followed by acid etching. The surface characteristics of machined and rough implants used in this study were examined by scanning electron microscopy as shown in Figure 1. Results revealed that the microtexturing protocol used in this study produced topographic changes in the surface to “rough” titanium implants creating more peaks and valleys, and increased pit formation.

In Vitro Establishment of *A. actinomycetemcomitans* Biofilm on Titanium Implants

To investigate the effects of microtexturing on the titanium implants for biofilm accumulation, the implants were inoculated in vitro with *A. actinomycetemcomitans* D7S-1 and cultured for 3 days. Scanning electron microscopy analysis shown in Figure 2 revealed agglomerates of coccoid-shaped *A. actinomycetemcomitans* cells scattered over the implants after 1 day of cultivation. After 2 days, the number and size of the agglomerates increased and more cells with coccoid to short-rod morphologies were observed. After 3 days of incubation, large areas of the implant surface were covered with multilayered bacteria of rod-shaped and tubular morphology, each 2 to 6 μ m in size, characteristic of well-established *A. actinomycetemcomitans* biofilms.^{27,28} This demonstrates that *A. actinomycetemcomitans* was capable of forming a biofilm on both machined and rough implants, but notably that rough implants showed greater mass of biofilm colonization and growth (Fig. 2). The viability of *A. actinomycetemcomitans* cells in a biofilm on rough implant surfaces was evaluated for periods up to 3 days of culture with live/dead staining and confocal laser scanning microscopy. Results revealed that 75% of biofilm bacteria were viable with uncompromised membrane integrity on rough implants on day 3, as demonstrated by green fluorescence (Fig. 3). In combined channels, live cells (green fluorescence) are in predominant number (Figs. 3C and 3E). Yellow fluorescence was noted

^{§§}Micro CAT II, Siemens Medical Solutions Molecular Imaging, Knoxville, TN.

^{|||}Amira, Visage Imaging, San Diego, CA.

^{¶¶}V-Works 4.0, Cybermed, Seoul, Korea.

around the inner aspects of biofilm clusters (Figs. 3B and 3E). These areas are composed of “dormant” biofilm (metabolically less active), which exhibits greater cell permeability, allowing the red fluorochrome to enter. In view of the sufficient accumulation of viable bacteria on *A. actinomycetemcomitans* biofilms, day 3 was selected for transfer of titanium implants to animals.

Clinical Results of *A. actinomycetemcomitans* Biofilm on Titanium Implants

A number of preliminary studies were carried out to determine the optimal oral sites and implant surface treatments. Initial surgical sites for implant placement included anterior palate and molar extraction sites (data not shown). Because of primary stability of the implants and technical purposes, implants were transmucosally placed to the center of the palate for short-term studies (1 to 14 days) and to the alveolar ridge for long-term studies (1 to 6 weeks). For our short-term investigation both rough and machined implants with or without established *A. actinomycetemcomitans* biofilm were transmucosally inserted into the center of the palate of rats. In vivo tissue response of rat mucosa after surgical placement of implants is shown in Figure 4. Clinical signs of inflammation, such as erythema, edema, and bleeding, started 2 days postoperatively on both machined (Fig. 4C) (five of six implants) and rough biofilminoculated implants (Fig. 4D) (six of six). In contrast, control non-inoculated implants, rough (Figs. 4B and 4F) (six of six implants) and machined, (Figs. 4A and 4E) (six of six implants) exhibited only slight postoperative signs of inflammation, which resolved within 7 days. The clinical signs of peri-implant mucositis, such as bleeding, progressed in biofilminoculated rats at day 7 (Figs. 4G and 4H) and beyond (Fig. 4). By 14 days, the implants inoculated with *A. actinomycetemcomitans* biofilm exfoliated leaving behind sockets filled with granulation tissue (Figs. 4K and 4L).

These results led us to experimentally investigate the effects of biofilm-inoculated rough implant placement in the alveolar ridge region and for a longer-term period (1 to 6 weeks), as illustrated in Figure 5. This surgical region was found to produce the greatest primary stability of implants, and to be able to support two implants per rat at one per side. Results in Figure 5 demonstrate clinical responses to biofilm-inoculated rough implants (Figs. 5B, 5C, 5E, and 5F) (six implants) and non-inoculated rough implants (Figs. 5A and 5D) (six implants) after 3 and 6 weeks. After 3 weeks, spontaneous bleeding and ulceration and necrosis were noted on tissues surrounding biofilm-inoculated implants (Figs. 5B and 5C) (four of five implants). At this stage, both biofilm-inoculated implant and control groups had one implant exfoliated. Moreover, the implant stability at 3 weeks was present in the control group (five of five implants) (Fig. 5A), and in biofilm-inoculated implants (four of five implants) (Figs. 5B and 5C). However, after 6 weeks post-implantation, sites were observed with exacerbating inflammatory responses, hyperplasia, and bleeding, and necrotic tissues were seen around experimental implants (Figs. 5E and 5F) and not around control implants (Fig. 5D). Implant stability was only evident in control sites (five of five implants) after 6 weeks (Fig. 5D), whereas biofilm-inoculated implants were mostly mobile (two of four implants) (Figs. 5E and 5F).

Microbial Characteristics of *A. actinomycetemcomitans*–Colonized Titanium Implants

The in vivo persistence of *A. actinomycetemcomitans* on the rough titanium implants was examined by PCR and culture after 1 to 14 days (data not shown) and after 1, 3, and 6 weeks post-implantation (Table 1). PCR detection for the presence of *A. actinomycetemcomitans* on the surface of implants placed in vivo was positive for samples from the biofilm-inoculated implants 1 week (six of six implants), 3 weeks (five of five remaining implants), and 6 weeks postoperatively (one of four remaining implants). Because some of the implants exfoliated, the microbial data refer to the remaining implants. Control non-inoculated implants (Table 1) and saliva samples (data not shown) were negative at all time points.

Cultivable *A. actinomycetemcomitans* was detectable on the surface of biofilm-inoculated implants (Table 1) after 1 week (six of six implants), 3 weeks (three of five implants), and 6 weeks (one of four implants) post-implantation. *A. actinomycetemcomitans* was not detectable on any of the control non-inoculated implants at any of the time points tested.

μCT Imaging Analysis of Peri-Implant Bone Responses to *A. actinomycetemcomitans*–Biofilm Titanium Implant

To gain perspective into the effects of *A. actinomycetemcomitans* biofilm on peri-implant bone, μCT imaging was performed on rats after 3 and 6 weeks post-implantation (Fig. 6). Representative two- and three-dimensional reconstructed μCT images demonstrate visual evidence for peri-implant bone loss around *A. actinomycetemcomitans* biofilm-inoculated implants 6 weeks post-implantation (Figs. 6B, 6D, and 6F). In comparison, uniform bone remained around control implants (Figs. 6A, 6C, and 6E). To quantitate peri-implant bone, a virtual cylindrical divider was placed around all implants within three-dimensional reconstructed images. The proportion of bone volume occupying these cylindrical spaces was measured and compared (Fig. 6G). Comparison of control ($53.9\% \pm 10.1\%$) and biofilm-inoculated ($38.6\% \pm 7.2\%$) implants at 3 weeks was not statistically significant. At 6 weeks, significantly lower ($P < 0.05$) proportion of biofilm-inoculated ($29.6\% \pm 7.6\%$) peri-implant space was occupied by bone compared to control ($50.5\% \pm 9.6\%$) implants (Fig. 6G).

DISCUSSION

Accumulating evidence suggests that between 65% and 80% of microbial diseases go through a necessary biofilm phase.⁹ Biofilm-mediated inflammatory osteolytic oral infections, such as periodontitis and peri-implantitis, have complex etiology and pathogenesis and are thought to represent very similar conditions.^{4,29,30} The lack of animal models to examine and characterize infectious periodontitis and periimplantitis that accurately recapitulate the clinical biofilm-mediated condition has hampered the development of effective diagnostics and therapeutics. Such a model system requires implantation of a specific etiologic agent to successfully establish a pathogenic biofilm, whereby the interactions among the pathogen, the host, and the disease can be investigated. A number of obstacles have limited development of an effective animal model, including: 1) exogenous pathogenic bacteria have been typically transferred in their planktonic form and have difficulty competing with the indigenous biofilm to establish the required pathogenic biofilm; 2) previous studies have often required suppression of the indigenous flora by antibiotic pretreatment and multiple inoculations;^{31,32} and 3) when anaerobic bacteria are introduced in the oral cavity they have to colonize and survive in the supragingival environment, which is incompatible with their requirement of survival. To that end, the present experimental model was developed to transfer an established biofilm of a well-defined organism into animals leading to an inflammatory lesion with concomitant tissue destruction. This study presents preliminary support that this model overcomes some of the limitations highlighted by previous studies. In the present model, an established biofilm is transferred to obviate the need for planktonic bacteria to create a biofilm *de novo*. Additionally, the biofilm-inoculated implants are placed transmucosally, where the established biofilm is placed in submucosal space, which is akin to the subgingival environment. Moreover, antibiotic pretreatment and multiple inoculations were not necessary in the present study.

Unlike previous animal models that are ligature-based or involve inoculation of planktonic bacteria to induce the disease state,^{14–17} this model uniquely introduces established biofilms attached to titanium implants. It is important to note that this model was designed to investigate specific aspects of osteolytic biofilm-mediated diseases, such as periodontitis and

peri-implantitis, and not intended to recapitulate the entire disease process. As such, we introduced implants containing an established biofilm into the animals and did not seek to form *A. actinomycetemcomitans* biofilm *de novo* in the rats. This led to the persistence of *A. actinomycetemcomitans* in biofilms in most of the rats at 3 weeks post-surgery. After 6 weeks, one of four remaining implants continued to have detectable *A. actinomycetemcomitans* by PCR and had cultivable *A. actinomycetemcomitans*. At the present time, it is unclear whether the non-cultivable implants were the results of lack of viable bacteria or those that were in a persistor state.³³ It is possible that the lack of detection of *A. actinomycetemcomitans* is caused by sampling variability, because biofilm samples were taken from the very small implants using a toothpick. For future testing, implants will be removed and *A. actinomycetemcomitans* will be detected *ex vivo*. *In situ* detection of viable organisms using the live/dead assay in concert with specific *A. actinomycetemcomitans* detection with fluorescence *in situ* hybridization is also planned to address this question.

The microtexturing of titanium implant surfaces with grit-blasting and acid-etching led to significant increase in biofilm density on implants. This was favorable in biofilm formation in the present animal model. Currently, most dental implants in clinical use have a rough microtexture. The main reason for this surface topography has been to increase surface area to improve osseointegration. However, it is clear from the present study that the microtexturing of titanium implant surfaces also increases biofilm formation, which may potentially increase the inflammatory response.

The inoculation of *A. actinomycetemcomitans* was accompanied by clinical signs of peri-implant inflammation and bone loss, manifested in radiographic radiolucency detectable by μ CT, implant mobility, and eventual exfoliation of some implants. The exfoliation of implants in the present model is likely attributed to the fact that the biofilm was preformed on the entire implant surface before placement. One of the options that our group is currently pursuing to minimize implant exfoliation is the development of two-piece implants. Such novel miniaturized implants will allow surgical placement of sterile implants to facilitate their osseointegration with the host, which will be followed by the attachment of a transmucosal abutment with a preformed biofilm.

Aggregatibacter actinomycetemcomitans strain D7S-1 was selected as an initial experimental model because there is significant *in vitro* data with immune responses to this particular clinical strain and its deletional mutants.^{23,34–36} In the future, this novel animal model can be used for other biofilm pathogens, and complex multispecies biofilms, allowing the study of concomitant host responses because of introduced biofilm pathogens, and anti-biofilm treatment modalities. Any species or consortium of species capable of forming a biofilm could in principle be tested and in different animals using this model and delivery system.

One of the key determinants of *A. actinomycetemcomitans* colonization is the presence of fimbriae, which is mediated by expression of *tad* genes (tight adherence).^{37,38} *A. actinomycetemcomitans* isogenic mutants where *tad* has been knocked out have impaired ability to colonize and cause bone loss.³⁹ The *A. actinomycetemcomitans* strain D7S-1 used in the present report is fimbriated.^{31,40} However, it will be interesting to determine whether the smooth non-fimbriated variant also is able to persist on our implant biofilm model. Several lines of evidence have suggested that rats but not mice are an appropriate animal model to establish an indigenous *A. actinomycetemcomitans* infection because of the fact that: 1) *A. actinomycetemcomitans* is found as a natural inhabitant of some species of rats,^{41–43} whereas no reports of naturally occurring *A. actinomycetemcomitans* in mice have been found; 2) colonization of mice with *A. actinomycetemcomitans* is not predictable;⁴³

and 3) in vitro studies have demonstrated binding of *A. actinomycetemcomitans* to rat buccal epithelial cells and salivary-coated rat teeth.^{42–44}

CONCLUSIONS

The relationship among a specific oral biofilm pathogen, the host response, and tissue destruction is a necessary first step toward the future goal of mechanistic studies into biofilm-mediated conditions, such as periodontitis or peri-implantitis. Our clinical, microbiologic, and imaging results demonstrate that titanium implants that have established *A. actinomycetemcomitans* biofilms can be used as a biofilm delivery system by implanting transmucosally in the rat jaw where the *A. actinomycetemcomitans* persists as a biofilm, inducing osteolysis and tissue destruction. Investigating host and microbial gene expression, and their mutual interactions, will be feasible for the first time in this present model. Our current efforts have focused on characterizing the local and systemic immune responses to inoculation of the rats with an *A. actinomycetemcomitans* biofilm.

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Figure 1.

Scanning electron microscopy (SEM) of titanium implants. *A*) SEM image showing the head and part of the shaft of a machined implant used in the present study. Surface microtopography of machined (*B* and *D*) and rough (*C* and *E*) implants is illustrated.

Figure 2.

In vitro establishment of *A. actinomycetemcomitans* biofilm on titanium implants. Machined and rough titanium implants were incubated in culture media inoculated with *A. actinomycetemcomitans* D7S-1 for 1 to 3 days. SEM images of established biofilms on machined (**A through C**) and rough (**D through G**) titanium implants after 1 (A and D), 2 (B and E), and 3 (C, F, and G) days of culture.

Figure 3.

Viability of *A. actinomycetemcomitans* biofilm on the rough implant surface cultivated after 3 days. Implants with 3-day-old *A. actinomycetemcomitans* D7S-1 biofilms were sectioned at the head and shaft interface (A) before live/dead staining and visualized by confocal laser scanning microscopy. Green fluorescence indicates viable cells (C), red fluorescence shows cells with compromised membranes (D), and overlaid fluorescence channels are shown (B and E).

Figure 4.

In vivo tissue response of rat mucosa after surgical placement of implants. One implant was placed into the anterior region of the hard palate. Four different implant types were used, which included machined or rough and non-inoculated control or *A. actinomycetemcomitans* biofilm-inoculated implants. Clinical photographs of peri-implant tissues 2 (**A through D**), 7 (**E through H**), and 14 (**I through L**) days after surgical placement of titanium implants are illustrated. Representative clinical images showing the peri-implant mucosal tissues surrounding machined (**A, C, E, G, I, and K**) and rough (**B, D, F, H, J, and L**) titanium implants are shown. The implants consisted of non-inoculated controls (**A, B, E, F, I, and J**), and those with established *A. actinomycetemcomitans* biofilms (**C, D, G, H, K, and L**).

Figure 5.

In vivo tissue response of rat mucosa after surgical placement of implants. *A. actinomycetemcomitans* biofilm–inoculated or control non-inoculated microtextured implants were placed (one on each side) into the maxillary alveolar ridge. Both implants in each rat were similarly treated. Clinical photographs were taken of rats 3 and 6 weeks after surgical placement of rough titanium implants, control non-inoculated implants (**A** and **D**), or implants with established *A. actinomycetemcomitans* biofilm (**B**, **C**, **E**, and **F**).

Figure 6. μCT analysis of peri-implant bone volume around control and *A. actinomycetemcomitans* biofilm-inoculated implants. μCT two-dimensional (**A** and **B**) and three-dimensional (**C through F**) radiographic images of control (**A**, **C**, and **E**) and *A. actinomycetemcomitans* biofilm-inoculated implants (**B**, **D**, and **F**) at 6 weeks post-implantation. (**G**) Quantitative analysis of peri-implant bone volume after 3 and 6 weeks post-implantation is shown (n = 3). * $P < 0.05$.

Table 1Detection of *A. actinomycetemcomitans* Biofilm After Surgical Placement of Implants

Time	PCR		Culture	
	Control	Aa	Control	Aa
1 Week	0% (0/6) *	100% (6/6)	0% (0/6)	100% (6/6)
3 Weeks	0% (0/5)	100% (5/5)	0% (0/5)	60% (3/5)
6 Weeks	0% (0/5)	25% (1/4)	0% (0/5)	25% (1/4)

* Number of positive implants/total number of remaining implants