Animal Models to Study Host-Bacteria Interactions Involved in Periodontitis

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Abstract
Animal models have distinct advantages because they can mimic cellular complexities that occur in humans in vivo and are often more accurate than in vitro studies that take place on plastic surfaces with limited numbers of cell types present. Furthermore, cause and effect relationships can be established by applying inhibitors or activators or through the use of genetically modified animals. Such gain or loss of function studies are often difficult to achieve in human clinical studies, particularly in obtaining target tissue due to important ethical considerations. Animal models in periodontal disease are particularly important at this point in the development of the scientific basis for understanding the predominant pathological processes. Periodontal disease can be broken down into discrete steps, each of which may be studied separately depending upon the animal model. These steps involve the development of a pathogenic biofilm, invasion of connective tissue by bacteria or their products, induction of a destructive host response in connective tissue and limitation of a pair process that follows tissue breakdown. Animal studies can test hypotheses related to each of these steps, and should be evaluated by their capacity to test a specific hypothesis rather than recapitulating all aspects of periodontal disease. Thus, each of the models described below can be adapted to test discrete components of the pathological process of periodontal disease, but not necessarily all of them.

Disciplines
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Abstract

Animal models have distinct advantages because they can mimic cellular complexities that occur in humans in vivo and are often more accurate than in vitro studies that take place on plastic surfaces with limited numbers of cell types present. Furthermore, cause and effect relationships can be established by applying inhibitors or activators or through the use of genetically modified animals. Such gain or loss of function studies are often difficult to achieve in human clinical studies, particularly in obtaining target tissue due to important ethical considerations. Animal models in periodontal disease are particularly important at this point in the development of the scientific basis for understanding the predominant pathological processes. Periodontal disease can be broken down into discrete steps, each of which may be studied separately depending upon the animal model. These steps involve the development of a pathogenic biofilm, invasion of connective tissue by bacteria or their products, induction of a destructive host response in connective tissue and limitation of a repair process that follows tissue breakdown. Animal studies can test hypotheses related to each of these steps, and should be evaluated by their capacity to test a specific hypothesis rather than recapitulating all aspects of periodontal disease. Thus, each of the models described below can be adapted to test discrete components of the pathological process of periodontal disease, but not necessarily all of them.

Animal models have limitations. However, the limitations are often less severe than those encountered during in vitro experiments, in which cells are typically examined on a plastic surface. Moreover, animal models often allow a more definitive analysis of cause and effect relationships than human clinical studies. A critical feature of animal models is the ability to examine complex host-bacteria interactions that cannot be carried out using single-cell populations under artificial conditions. This may be true even in the absence of a periodontium. For example, the impact of cytokines on the recruitment of inflammatory cells and the induction of bone resorption has been shown to occur in many different tissues, of which the periodontium is one. Thus, one can study the potential involvement of cytokines in the bone resorptive process in tissues ranging from the calvarium to the periodontium. This is possible since the functional role of particular mediators is typically consistent regardless of the specific bone examined. This raises the point that the value of
animal studies should be considered in terms of their capacity to test specific hypotheses rather than their capacity to mimic all aspects of periodontal disease.

A problem in studying periodontal disease is the question of ‘chronic’ versus ‘acute’ nature of the inflammation. It is not known whether periodontal breakdown occurs in bursts or whether it is slow and continuous. At any point in time, the human periodontium adjacent to tooth has inflammatory cells recruited with characteristics of both chronic and acute inflammation. It is also clear that periodontal tissue breakdown involves both innate and acquired immunity [1–4]. The role of chronic and acute inflammation or innate versus acquired immunity can be studied using the appropriate animal model.

Animal models have characteristic features useful for investigating molecular mechanisms involved in the pathogenesis of periodontal diseases. Mouse models are particularly helpful because of the number of genetically modified strains that are available to study both gain of function and loss of function with regard to specific genes. Moreover, animal models provide an opportunity to investigate discrete steps of periodontal disease. Periodontal disease can be broken down into 5 different phases: (1) colonization; (2) invasion across the epithelium into connective tissue; (3) stimulation of an inflammatory response; (4) induction of connective tissue breakdown and bone resorption; (5) limitation of damage by repair processes, such as bone coupling, resulting in net bone loss. By selecting the appropriate animal model each of these steps can be investigated individually, whereas in human studies it is difficult to isolate a specific step and in vitro studies lack the complexity to examine specific phases. Moreover, a considerable amount is known regarding mouse and rat immune systems, and a wide range of reagents are available.

Rat Ligature Model

The induction of periodontal disease by the placement of ligatures around the teeth has been used in many different types of animals ranging from rats to nonhuman primates. Recently there has been considerable interest in the rat ligature model. Placement of a ligature leads to the accumulation of dental plaque and microulceration of the sulcular epithelium which, in turn, facilitate the invasion of periodontal pathogens into connective tissue. Loss of periodontal attachment and resorption of alveolar bone occurs predictably in a 7-day period in the rat [5–9], although some experiments have been conducted for much longer periods of time [10–13]. Similar to human periodontitis, bacterial penetration of the host tissue is thought to be a crucial factor affecting alveolar bone loss. The ligature alone does not initiate significant gingival inflammation or bone loss in gnotobiotic rats [12]. The role of bacteria in initiating periodontal destruction in the rat ligature model is supported by topical application of antiseptics, which reduce alveolar bone resorption, and systemic application of antibiotics, which reduces loss of attachment and bone resorption [11, 14, 15]. The role of bacteria in this model is further supported by findings that osteoclastogenesis and alveolar bone resorption are enhanced by the application of gram-negative bacteria [16].

As in human periodontitis, the stimulation of a host response induces periodontal destruction in the rat ligature model. This response involves the formation of gingival inflammation prior to bone resorption [17]. Periodontal destruction is decreased if the host response is reduced by inducing endotoxin tolerance [16]. The role of the inflammatory response is further demonstrated by reduced gingival inflammation, osteoclast formation and alveolar bone loss in the rat ligature model with application of prostaglandin inhibitors, including COX-2 specific inhibitors [7]. Low-dose doxycycline also reduces alveolar bone loss by inhibiting MMP activity [6]. In contrast, application of the cytokines interleukin (IL)-1 or TNF enhances bone loss in this model [18–22]. The ligature-induced model is also found to be sensitive to some systemic effects, such as smoking. Ligated rats exposed to cigarette
smoke have greater bone loss and higher MMP-2 activity compared to animals with experimental periodontitis who were not exposed to cigarette smoke [23]. Similar results have been obtained when injecting nicotine into ligated rats [10, 24].

The use of the rat ligature model is useful in examining the effects of systemic disease on periodontal disease progression. For example, the impact of diabetes can be investigated by using type-2 Zucker diabetic fatty (ZDF) rats. ZDF rats develop diabetes by 8 weeks of age due to obesity and insulin resistance [25]. With ligature-induced periodontitis, greater periodontal bone loss is found in the ZDF rats compared to matched normoglycemic controls. This is explained by the fact that diabetes enhances the duration and intensity of the inflammatory infiltrate, increasing osteoclastogenesis and thereby augmenting alveolar bone loss [26]. Furthermore, the effect of diabetes on bone coupling and its contribution to periodontal disease may be investigated after removing the ligatures. In this case, the amount of new alveolar bone formed following bone loss was 2.4- to 2.9-fold higher in normoglycemic rats compared with ZDF (diabetic) rats. The mechanisms for diminished bone coupling in diabetic animals may be explained by fewer bone lining and periodontal ligament cells, due in part to diabetes-enhanced apoptosis. Therefore, the rat ligature model is useful in investigating mechanisms by which a systemic condition such as diabetes affects the periodontium.

**Rat A. actinomycetemcomitans Infection Model**

An important aspect of periodontal disease is bacterial colonization of the tooth surface and its invasion into connective tissue. A problem with many rodent models of periodontal disease is that the bacteria used to induce the disease process only transiently infect the oral cavity since rodents are not natural hosts for many human bacteria. A well-documented exception to this general principle is infection of the rat with Aggregatibacter actinomycetemcomitans. Known natural hosts of A. actinomycetemcomitans include humans, old-world monkeys, cows and rats [27]. However, caution is still warranted since many laboratory strains of A. actinomycetemcomitans have a ‘smooth’ phenotype and greatly diminished capacity to adhere to surfaces, which is in sharp contrast to the A. actinomycetemcomitans found in fresh clinical isolates [28]. Therefore, the utility of bacterial laboratory strains are open to question, just as results from immortalized mammalian cells may need to be confirmed with primary cell cultures. This point is illustrated when the capacity of the smooth strain of A. actinomycetemcomitans to infect the oral cavity was found to be limited compared to recent clinical isolates [29]. Since A. actinomycetemcomitans naturally colonizes the oral cavity of rats, it can be examined to establish virulence factors critical for colonization [30, 31].

Wild-type A. actinomycetemcomitans adheres to rat buccal epithelial cells and is a common inhabitant of rice rats, while it is difficult to find naturally in Sprague Dawley rats from commercial sources [27]. However, A. actinomycetemcomitans can colonize the latter. On the other hand, A. actinomycetemcomitans has difficulty infecting the oral cavity of mice. Therefore, the mouse is not an adequate model to study A. actinomycetemcomitans colonization. Studies with A. actinomycetemcomitans-induced bone loss in mice are apparently due to transient infections that are self-limiting.

Several important factors should be considered when assessing colonization, including any reduction in the existing flora. The existing oral flora should be reduced to facilitate the addition of an exogenous bacterium. It is advantageous to tag A. actinomycetemcomitans as a means of distinguishing it from the existing flora, for example by using a rifampicin-resistant strain. To avoid transfer of microorganisms from one animal to another, they should be housed in separate cages. One method to consistently inoculate the oral cavities of
rats with *A. actinomycetemcomitans* is to include known amounts in food, as described by Fine et al. [29].

The *A. actinomycetemcomitans* model has also been used to examine periodontal bone resorption and the systemic host response to infection. Li et al. [32] examined the role of B and CD4+ T cells in adaptive immunity of rats infected with *A. actinomycetemcomitans*. Results showed that elevated lymphocyte counts in rats inoculated with *A. actinomycetemcomitans* and the number of B and CD4+ T cells from regional lymph nodes were increased. After 2 weeks, *A-actinomycetemcomitans*-specific IgG and IgG2 responses were significantly enhanced. mRNA levels of several cytokines were found to be elevated in the lymph nodes including IL-2, IL-1, TNF, CD40 ligand, FasL, RANKL and osteoprotegerin.

The *A. actinomycetemcomitans* oral inoculation model in the rat has been used to examine the impact of rat strains on colonization and alveolar bone loss, in particular the fawn-hooded hypertensive (FHH), Dahl/salt sensitive and Brown Norway strains. Results showed significantly higher *A. actinomycetemcomitans* colonization in FHH rats than in the other strains. Interestingly, *A. actinomycetemcomitans*-associated bone loss was only observed among the FHH strain.

**Oral Gavage Model of Experimental Periodontitis**

The introduction of human strains of bacteria by oral gavage (inoculation of bacteria into the oral cavity) and subsequent impact on the periodontium has been studied in various rodent models [33, 34]. Various bacterial strains associated with periodontitis in humans have been used in this model, including *Porphyromonas gingivalis* [3, 35–37], *A. actinomycetemcomitans* [38], *Tannerella forsythia* [39] and *Treponema denticola* [40]. Typically, mice or rats are given a known number of bacteria in a viscous suspension (2% carboxymethylcellulose) administered orally. In many cases, the oral infection by human bacteria is transient. PCR has frequently been used for detection of bacteria, which has a limitation in that the bacteria detected are not necessarily living. Nevertheless, *T. denticola* has been detected in mice 71 days after inoculation [40] and *P. gingivalis* has been detected at up to 11 weeks [41]; 45% of rats exposed to *P. gingivalis* and 80% exposed to *T. denticola* or *T. forsythia* were found to harbor these bacteria after 4–6 weeks [42]. Reproducibility and predictability of infection is enhanced by prior treatment with antibiotics to reduce the endogenous oral flora and with repeated inoculations of the organism. Typical inoculations are scheduled every other day for 1 week with 10⁹ CFU, although the number of inoculations may be increased in mouse strains that are less susceptible to periodontal disease. Significant bone loss can be measured histologically, by macroscopic analysis or by micro-computed tomography. Alveolar bone resorption is usually assessed around the maxillary molars since induction of bone loss in the lower molars is slower due to the thicker cortical alveolar bone and wider buccolingual dimensions. In many reports, mice are euthanized 6 weeks after the final inoculation [3, 43, 44], although recent reports have shown alveolar bone loss can be detected as early as 2 weeks after the final oral challenge with bacteria or 3 weeks after initiating infection [45]. It has been reported that inoculation of bacteria induces an immune response that may be comparable to that associated with periodontal disease in humans. Migration of leukocytes (mononuclear cells and neutrophils) into the gingival connective tissues, proliferation of the junctional epithelium, increased vascularization and serum antibody (IgG1 and IgG2) levels against the periodontal pathogens have been reported in these models [41]. Increased inflammatory cytokine levels have been reported following bacterial inoculation, including elevated levels of TNF-α, IL-12 and IFN-γ [46] and decreased anti-inflammatory cytokines such as IL-10 [40].
Both rats and mice are susceptible to alveolar bone loss induced in the oral gavage model. However, there are differences in the susceptibility to experimental periodontal disease among the various strains of mice. BALB/c, AKR/J, DBA/2J and C3H/HeN mice are more susceptible than C57Bl/6, A/J, 129/J, SJL/J and C3H/HeJ [36, 47]. These differences are related to genetic variances among the strains affecting components of the immune response, such as the point mutation on Toll-like receptor 4 (TLR4) in C3H/HeJ mice or differences in adaptive immunity [48]. Normal genetic variation (as well as genetically engineered alterations) in mice provides a potentially rich avenue to establish a cause-and-effect relationship between periodontal disease and aspects of the host response. Similarly, it would be interesting to take advantage of different characteristics of different rat strains to study particular pathogenic aspects of periodontal diseases, such as deficiencies in innate or adaptive immunity and the hyperinflammatory phenotype.

Infection of the oral cavity by topical administration of bacteria has also been carried out in rats. Many of these studies have examined the Sprague Dawley strain [41, 42, 45]. There is some evidence that genetic variances in laboratory rats influence the pathogenesis of periodontal disease and may be used to investigate the possible interrelationships between periodontal disease and the host response, such as differences in Th1 cytokine expression between Lewis and Fisher 344 strains [49].

One aspect that has been discussed in the oral infection model is the use of a single bacterial species versus the use of two or more microorganisms associated with periodontal disease. The complexity of bacterial stimulation is supported by findings that the persistence of *P. gingivalis* in the oral cavity of rats 4 weeks after initial challenge is significantly increased from 45 to 80–100% when this bacterium is co-infected with *T. forsythia* and *T. denticola*. Alveolar bone loss is significantly greater in animals challenged with a polymicrobial oral infection rather than monoinfection [42, 50].

It should be kept in mind that the inoculated bacterium is not necessarily responsible for periodontal bone loss since the introduction of exogenous bacteria may alter the host's bacterial flora. Unless one works with germ-free animals, specific pathogen-free laboratory rodents are not necessarily free of bacteria that can induce periodontal breakdown. This reflects the current understanding that there is a fine line between harmless and opportunistic commensal bacteria that can stimulate destruction of the periodontium. Moreover, there are complex bacterial interactions that are not always apparent. For example, exogenous infection with a single species of periodontal pathogen like *P. gingivalis* may modify the oral biofilm of the animal by interacting with endogenous bacterial species. The alteration of the endogenous flora may contribute to the disease process rather than the direct action of *P. gingivalis*.

As with all other experimental models of periodontal disease, variations of the oral gavage model need to be considered in regard to the purpose of the study. The oral gavage model has been used to establish proof of principle in vaccine development and to examine the relative impact of different antigens of pathogenic bacteria. For instance, immunization against RgpA and Kgp gingipain peptides or intact RgpA gingipain reduces *P. gingivalis*-induced bone resorption [51, 52]. The oral gavage model has been used to mechanistically link *P. gingivalis*-induced periodontitis to systemic conditions. For example, oral infection of hyperlipidemic mice with *P. gingivalis* mice accelerates atherosclerotic plaque formation [53, 54] and evidence suggests that this microorganism may play a role in aortic aneurysms through a mechanism involving smooth-muscle-cell-dependent hyperplasia [55]. However, the oral gavage model in rodents may be limited in studying several important aspects of human periodontal disease, such as the colonization process or bacteria-bacteria interactions.
Lipopolysaccharide Injection Model

Gram-negative bacteria are thought to be important periodontal pathogens. The lipopolysaccharide (LPS) component of the cell wall of these microorganisms is a significant inflammatory stimulus that triggers an innate immune response. Thus, the injection of LPS into the gingival tissues is a model for examining how the innate immune response to this bacterial component induces inflammation to stimulate osteoclastogenesis and bone loss. This model produces a histopathological aspect similar to the other models and to that observed in established periodontitis in humans, characterized by increased infiltration of leukocytes, higher levels of proinflammatory cytokines, collagen degradation and alveolar bone resorption. Typically, a defined amount of purified bacterial LPS suspended into small microvolumes (1–6 μl) is injected into the gingival tissues surrounding the posterior teeth of either mice or rats. The technique is somewhat sensitive and requires the use of microsyringes for small volumes and thin needles (28–33 G). When performing injections in mice, the use of optical magnification is suggested to improve visualization. The injection site frequently used is the palatal aspect of the upper first molars, but some studies have also performed the injections on the interdental papilla between lower first and second molars [56, 57]. These injections are performed under general anesthesia, usually 3 times a week for the experimental period. Alveolar bone loss has been observed as early as 7 days after the start of injections of LPS from various microorganisms, including Escherichia coli [58], A. actinomycetemcomitans [47] and Salmonella typhimurium [56], suggesting that the source of LPS may not significantly affect this particular outcome. Most studies have used experimental periods of 3–8 weeks for both mice [59, 60] and rats [61, 62].

The origin of the LPS used, both in terms of the microorganism from which it was obtained and of the purification process, is an important variable to be considered. Even though LPS from most bacteria are considered activators of TLR4, many periodontopathogenic bacteria – including T. forsythia, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum, A. actinomycetemcomitans, Veillonella parvula and P. gingivalis – also activate TLR2 [63, 64]. In fact, the ability of P. gingivalis to induce alveolar bone loss is nearly abolished in mice lacking TLR2 [65]. However, TLR4 may also play a prominent role in stimulating bone resorption as the C3H/HeJ mouse strain, which lacks functional TLR4, has significantly less alveolar bone resorption than wild-type mice upon injections of A. actinomycetemcomitans LPS [47]. A direct comparison between A. actinomycetemcomitans and P. gingivalis LPS suggests that both stimulate inflammation and bone loss, but the latter is a little bit less potent than A. actinomycetemcomitans LPS [57]. In a similar model, injections of E. coli and P. gingivalis LPS were compared by injecting them into the scalp to stimulate inflammation and calvarial bone resorption. In this model, P. gingivalis LPS and E. coli LPS were both relatively potent in stimulating inflammation and bone loss at the site of injection, but E. coli LPS was more potent in stimulating systemic inflammation resulting from endotoxemia [66].

Depending on the purpose of the study, the purification process for obtaining LPS is also an important concern, since possible contamination with proteins or DNA may trigger activation of other receptors and pathways leading to inflammation and alveolar bone loss. This concern may be particularly important for ‘home made’ preparations of LPS. The bacterial strain, purification methods and even culture conditions may influence the biochemistry of LPS. The antigenic moiety of LPS is contained in its lipid region, termed lipid A. Variations in the lipid A region can modulate the immune response elicited and the pathogenic potential of the LPS [67].

Among the advantages of various injection models is the greater experimental control over the pathogenic stimulus, which is directly delivered to the tissues (i.e. no ‘colonization
variable’) in a titratable manner. Several mediators may be studied, such as specific TLR agonists to investigate proof of principle regarding the role of TLRs in inflammation and tissue destruction. Alternatively, live or heat-killed bacteria can be injected to investigate host-bacteria interactions in the periodontium. These mediate or induce tissue destruction by activating different cell types present in the host tissues, including fibroblasts, lymphocytes, macrophages, dendritic cells and osteoblasts. Events stimulated downstream include the production and release of an array of inflammatory mediators by these activated cells, including prostaglandins, cytokines and MMPs. Thus, injection models may be useful for investigating the activation of signaling pathways.

Calvarial Model

The calvarial model was first designed and developed to study the effect of cytokines on osteoclastogenesis [68]. It has been employed to investigate the effect of bacteria on bone resorption [69], bone resorbing activity of dendritic cells [70] and host-bacteria interactions in connective tissue in vivo [71, 72]. In this model, the stimulus is injected into the subcutaneous connective tissue over the calvarium. Histologically classic inflammatory events occur, including the rapid expression of proinflammatory cytokines within a few hours and the recruitment of polymorphonuclear leukocytes (PMNs) within 24 h [73]. Bone resorption is induced within 3–5 days depending upon the size of the stimulus [74, 75]. If the stimulus is small, there is an inflammatory event which resolves with relatively little tissue destruction. A large inoculum creates a perturbation that induces a soft tissue wound and considerable bone resorption that subsequently undergoes repair. New bone formation can then be examined as part of the healing process and can be measured 8–12 days following inoculation of the stimulus [74, 76, 77]. The precise time frame in which events are measured may be modified by the strain of mouse [78]. The calvarial model is useful in examining the sequence of inflammation, destruction and repair. This may be critical to study periodontal disease where the net loss of tissue may be related to loss of balance between destruction of bone and connective tissue and the degree of repair that occurs.

An advantage of the calvarial model is that a sufficient amount of tissue is obtained for various profiling techniques. In one example, P. gingivalis, T. denticola and T. forsythia were injected into the connective tissue overlying calvarial bone in mice, and mRNA profiling was performed. This led to the identification of a number of inflammatory mediators that were induced in a bacteria-specific pattern [79–81].

The calvarial model is also well adapted to examine cause-and-effect relationships. In one example, the role of TNF-α in mediating the effects of P. gingivalis infection were examined by comparing TNF-receptor-ablated mice with wild-type controls. It was determined that formation of PMN infiltrate and osteoclastogenesis was dependent upon the induction of TNF receptor signaling. This suggests that the TNF activity plays an essential part in the inflammatory infiltrate formed in response to P. gingivalis. Although many bacterial components are chemotactic for PMNs, they are assumed to be less significant than bacteria-induced expression of cytokines, particularly TNF. In the same example, TNF activity also played an important role in the stimulation of fibroblast cell death stimulated by P. gingivalis. When P. gingivalis was inoculated in TNF-receptor-deficient mice, a 75% reduction in fibroblast apoptosis and a 60% decrease in osteoclast formation resulted compared to the same inoculation in wild-type mice [72]. A similar approach was used to establish the importance of TLR signaling pathways in response to LPS [77] and to demonstrate that dendritic cells have an important role in bone-resorption-associated inflammatory stimuli [70]. Likewise, the calvarial model has been used to investigate the functional importance of apoptosis in bacteria-induced bone resorption [76, 82]. When apoptosis was limited, there was a much more robust healing response to a large bacterial
inoculum, particularly in diabetic animals. The anabolic effect of fluoxetine following bone resorption was demonstrated using the calvarial model [78]. Thus, after an episode of bone resorption, fluoxetine induced greater reparative bone formation, i.e. bone coupling, than that seen in vehicle-treated mice. This study also illustrates the necessity of using histological methods of analysis to better understand mechanisms. When examined by micro-computed tomography alone, it was not clear whether fluoxetine diminished bone resorption or enhanced coupled bone formation. According to the histological analysis, the more dominant effect was upon the coupled bone formation.

The other benefit of using the calvarial model is the capability to deliver a precise amount of inoculum and examine qualitative changes in the response to different levels of the same stimulus. This has been studied in the calvarial model using mice with targeted deletions as well as inhibitory antibodies. It has been indicated that osteoclastogenesis is induced by high doses of enteric LPS and substantially mediated by IL-1 and TNF receptor signaling. On the other hand, lower doses of LPS induce osteoclastogenesis through other pathways that are independent of TNF and IL-1 and involve IL-11 [83, 84].

In addition to studying bone repair, soft tissue healing can also be investigated in the calvarial model. The microarray studies mentioned above indicated that the gene transcription profiles in inflamed calvarial bone induced by microbial infection were different from the profiles observed in the soft tissue [79–81]. Another study has demonstrated the use of this model to examine a bacterially induced soft tissue wound that follows the classic steps of healing, including the migration of fibroblasts into the wounded site, proliferation, production of extracellular matrix and reorganization [85]. The repair of bacteria-associated wounds in diabetic mice is related to the greater production of TNF. By use of the calvarial model, it was shown that a TNF-specific inhibitor considerably enhanced the repair process following injection of P. gingivalis [76]. Indeed, there is a significant improvement in soft tissue healing when fibroblast apoptosis is reduced with a caspase inhibitor [82]. This may yield further insights into the periodontium, where the loss of fibroblasts is one of the most distinctive cellular changes that occurs in progressive periodontal disease [86]. Thus, studies in the calvarial model point to potential novel differences in the response to a bacterially induced injury in diabetic animals.

As mentioned above, the capacity to control an infection by inoculation allows precise examination of the induced host response. This can be further enlarged to study the effect with or without the presence of the adaptive immune response. Mice are naïve to P. gingivalis. By immunizing mice before injection of bacteria into the calvarial connective tissue, the contribution of the adaptive immune response can be examined by comparison with non-immunized mice [71]. A striking feature following P. gingivalis inoculation was the enhanced expression of bone-resorptive cytokines, osteoclastogenesis and bone loss in the immunized group, suggesting that the adaptive immune response contributes to the destruction of bone stimulated by P. gingivalis. Moreover, reparative coupled bone formation was significantly inhibited by the addition of the adaptive immune response [87].

**Rat Mandibular Critical Size Defect**

Knowledge of bone formation is important in understanding periodontal disease and treatment. It has been proposed that a failure to form bone after an episode of periodontal bone resorption (from uncoupling) is an important component of periodontitis [88]. Therefore, models of bone formation are important in discussing the spectrum of various periodontal animal models. The rat mandibular bone defect model was developed to study bone formation in the mandible [89]. This model has been useful in designing studies to compare the efficacy of different materials to augment bone formation including barrier...
membranes [90–92], bone grafts [93], growth factors and hormones [92, 94–96] and other factors such as ultrasound stimulation [97, 98]. In this model, the angle of the rat mandible is surgically exposed on both the buccal and the lingual aspects and a through-and-through osseous defect of 5 mm is created with a trephine burr. A similar defect may be created on the contralateral side, which may be used as a control if specific bone augmentation material is being tested.

In recent years, micro-computed tomography has been used as a fast precise nondestructive analytical procedure to measure bone volume within the defect created. There is a significant correlation (p < 0.0001) between micro-computed tomography values and histomorphometric analysis in measuring the amount of new bone and grafted material after 3 weeks of early healing [99, 100]. The rat mandibular bone formation model has been used to compare the performance of a degradable synthetic barrier membrane PDLLCL [poly(D, L-lactide- e-caprolactone)] with a comparable type 1 collagen or expanded ePTFE (polytetrafluoroethylene) membrane [101]. It was found that significantly more bone formation was observed under type 1 collagen and ePTFE membranes than the PDLLCL membranes. This model has been used to compare bone grafting materials β-tricalcium phosphate (β-TCP), allogenic bone and allogenic bone combined with β-TCP [93]. In this study, the authors concluded that β-TCP absorption occurred at a slower rate and enhanced bone formation when combined with an allograft because it helped maintain space more effectively than the allograft alone.

One of the advantages of the mandibular-angle-defect model is the ability to investigate the efficacy of locally and systemically delivered biologics. Local delivery of growth factors has been investigated in the rat mandibular defect model to compare bone morphogenetic protein-2 (BMP-2) with and without barrier membrane [94]. Interestingly, the results indicated that BMP-2 without membrane had significantly more bone repair than BMP-2 covered with membrane. It was suggested that the barrier membrane blocked a source of stem cells from migrating into the defect and limiting the amount of new bone formation. Since then, other studies with a growth factor have shown similar tendencies, which has led to recommendations that biologics which enhance bone formation should not be used with barrier membranes. This model has also been used to evaluate the effectiveness of systemically delivered calcitonin in promoting bone growth in surgical bone defects in rat mandibles [96]. The results revealed the calcitonin reduced bone formation at 1, 2 and 3 weeks of healing compared to the non-calcitonin-treated group. However, no significant difference was seen after 4 weeks of healing. Thus, bone repair in the calcitonin-treated animals appeared to be delayed in comparison to controls. Thus, the rat mandibular critical size defect is useful and effective in assessing factors and materials that promote bone formation.

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