

Rat gingival model for testing drugs influencing inflammation

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Abstract: Preclinical drug testing is an important area in new drug development where animals are used. An ideal animal model for this is one which is simple, reliable and can be extrapolated to humans. Topical drugs for inflammation are conventionally tested on the skin of animals after induction of inflammation. A gingival model would be simple as inflammation can be induced naturally by the action of plaque. Rats are a popular animal model for testing drugs as well as to study various diseases of the periodontium. Periodontal disease including gingival inflammation develops in rats in relation to indigenous plaque or experimentally induced bacterial products. A number of features of rats ranging from anatomy, histology and response to bacterial insult can be seen mirrored to a great extent in humans. There is a lot similarity in the development and resolution of inflammation as well as the gingival wound healing of rats and humans. This paper tries to explore the feasibility of using the rat gingival model for preclinical testing of drugs acting on or influencing inflammation and concludes by identifying potential areas of research using this model. The addition of such a simple and inexpensive model for preclinical testing of drugs will be welcomed by the drug developers.

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Introduction

A suitable animal model which is simple, fast and reliable and more importantly, that can be extrapolated to humans, is very much sought after in any drug testing. Periodontal research has favored many animal models of which the rat periodontal model is most popular for studying periodontal pathology. The pathogenesis of periodontal disease though bacterial initiated is mostly governed by the host factors. Apart from this, most of the events which occur are related to inflammatory

pathways. The rat periodontal model became popular as researchers believed that there is a lot of similarity in the development and resolution of inflammation as well as the gingival wound healing of rats and humans. This leads us to propose the use of this rat model to the area of preclinical drug testing. There are many features in a rat gingival model which will be favorable to drug discoverers for evaluating their findings. A few of these issues are discussed in this paper.

The paper describes the similarities in rats and humans followed by a critical review of how the rat gingiva can be a suitable model for testing topical drugs acting on inflammation. The paper concludes by identifying areas of research which can be explored in the use of a rat gingival model for pre-clinical drug testing.

The most frequently used rat strain in periodontitis studies is the Sprague-Dawley strain, but other strains have also been used successfully. The rat is equipped with one incisor and three molars in each quadrant.^{1,2} The structure and organization of the periodontal tissues of the molars (oral gingival epithelium, oral sulcular epithelium, junctional epithelium, periodontal collagen fibers, acellular and cellular cementum, and alveolar bone) are similar in rats and humans.^{2,3,4} Molars are fully erupted when the rats are 5 weeks old.⁵ There is migration of the teeth and the alveolar bone is continuously being remodeled.⁶

Periodontal Disease

Periodontal disease develops in rats in relation to indigenous plaque,^{7,8} to experimentally introduced microorganisms, or to experimentally introduced bacterial products.^{9,11} Destructive periodontal disease can also be induced in rats by placing a ligature around the cervix of a tooth.¹² Periodontal destruction occurs rapidly in rats. The clinical and histological findings in experimental periodontal disease in rats are similar to findings in man.¹³ Clinically, gingival bleeding upon gentle probing can be seen in rats a few days after the introduction of periodontal pathogens.¹⁴ Histologically,

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the junctional epithelium gradually undergoes pathologic changes, including rete peg formation, ulceration, and apical migration of epithelial attachment.^{10,15,16} An inflammatory cell infiltrate containing lymphocytes, macrophages; polymorphonuclear leukocytes (PMNs) appear in the connective tissue,^{14,16-23} and PMNs migrate through the epithelium into the gingival sulcus^{17,18}. Plasma cells are inconspicuous in the early stages of the disease,^{18,22} but with time they become very prominent.²⁴ Damage to collagen fibers¹⁷ and fibroblasts²⁵ also occurs. High osteoclast activity is seen after inoculation with Gram-negative organisms^{19,21,26} and lipopolysaccharide (LPS)^{9,27}. Significant bone destruction has been reported days after inoculation^{21,28}, and the lesions progress considerably between 60 and 90 days after infection.^{18,19, 29}

Cytokine Profiles

As cytokines are widely used to study the various effects of drugs, this section attempts to determine whether the profiles in humans and rats are similar.

Cytokines present in human gingiva

Cytokines are small soluble proteins produced by a cell that alter the behavior or properties of another cell locally or systemically. Included in the cytokine molecule group are interleukins, interferon, growth factors, cytotoxic factors, activating or inhibitory factors, colony stimulating factors, and intercrines. Thus, cytokines play an important role in numerous biological activities including proliferation, development, differentiation, homeostasis, regeneration, repair, and inflammation. At present, the mechanisms by which cytokines act on the target cells are classified into four types: autocrine, intracrine, juxtacrine, and paracrine.³⁰ For tissue homeostasis, a primary role can be ascribed to cytokines which are constitutively secreted by resident cells composing the tissue. On the other hand, in diseased states, cytokines may be secreted not only by resident cells but also by locally infiltrated immunocompetent cells.

Clinically healthy gingival tissues express a variety of growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-1 (TGF-1). The inflammatory cytokines such as IL-1, IL-6, and TNF- α are also detected in the clinically healthy gingival tissues, although their densities are relatively low compared with that in the inflamed sites. This suggests that a myriad of cytokines may be involved in the maintenance of periodontal tissue turnover or integrity. There is abundant evidence that cytokines are secreted by fibroblasts³¹, endothelial and epithelial cells. Gingival fibroblasts secrete a variety of cytokines (IL-1, IL-13, IL-6, IL-8, and TNF- α) and chemical mediators. Normal and inflamed gingiva express IL-13, IL-6, TNF- α , TGF- β 1 and IL-8.³² The cytokine profiles of epithelial cells from normal and inflamed gingiva are similar. Blood-borne fibroblast growth factor (bFGF) is seen in the gingival lamina propria and is also stored in intercellular spaces of gingival epithelial cells. TGF- β 2 is strongly expressed in the gingival epithelium; especially in the oral gingival epithelium.³³ IL-6 levels in inflamed gingival tissues were higher than those in healthy tissues.³⁴

Cytokines expressed in gingiva of rats

Various cytokines were expressed in normal healthy gingiva as well as in LPS induced gingival tissues of rats and mainly include IL1, TNF α and IL8.^{32,35,36} Interleukin 1beta (IL-1beta), tumour necrotic factor-alpha (TNF-alpha), and interferon-gamma (IFN-gamma) play an important role in inflammation, while platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-beta) and blood-derived fibroblast growth factor (bFGF) are the most important growth factors found in periodontal tissues. The concentrations of these cytokines in normal rat periodontium were lower in number compared to in inflammatory condition.³⁷

In the normal rat periodontium, TNF α , IL-1 α and IL-1 β positive cells were mainly detected in the coronal half of the junctional epithelium (JE) especially in the superficial layers which faces the tooth surface and

gingival sulcus. The number of positive cells increased in an apicocoronal direction. JE cells play an important role in the first line of defense against LPS challenge and the proinflammatory cytokines transiently produced by host cells may be involved in the initiation of inflammation and subsequent periodontal destruction. Small numbers of TNF α and IL1 β positive cells are also seen in oral gingival epithelium (OGE) and oral sulcular epithelium (OSE). A small number of TNF α positive cells was present in the gingival connective tissue and osteoblasts lining the outer surface of alveolar bone crest. Epithelial rest cells of Mallasez only stained positive for IL1 β .³⁷

Topical lipopolysaccharide (LPS) application was done on the tooth and subgingivally in rats to provoke initial periodontal destruction and studied as a periodontitis model. After application of LPS, not only fibroblasts, neutrophils, and macrophages were present in the gingival connective tissue, but also almost all the JE cells expressed TNF- α , IL-1 α , and IL-1 β . The cytokine-positive epithelial cells were also increased in the gingival sulcus.³⁷

In the gingival epithelium, many epithelial cells were strongly positive for TNF- α within 3 hours of LPS induction. The superficial layers of JE as well as the deep layers were positive for TNF- α . During this early phase, neutrophils infiltrated into the JE and its subepithelial area, and macrophages and most of the fibroblasts in the connective tissue subjacent to the JE were also positive for TNF- α . The expression pattern of IL-1 α and IL-1 β in the gingival tissue was similar to that of TNF- α .³⁷

After 1 day of LPS application, the gingival epithelial cells intensively stained positive for TNF- α , IL-1 α , and IL-1 β cell and spreaded in a more apical direction but after the second day there was a gradual reduction in the cytokines especially in the apical part of the JE. The expression of TNF- α in macrophages and gingival fibroblasts was enhanced. TNF- α positive fibroblasts and osteoblasts were seen in the crestal area, and in deeper regions of the periodontal ligament. TNF- α was also expressed in osteoclasts and preosteoclasts that increased along the alveolar bone margin in this

period. Such expression of proinflammatory cytokines in the periodontal tissues was maintained until 3 days after LPS application and showed a decline. At 7 days, the number of the cytokine-positive infiltrating and resident cells in the periodontal tissues decreased to a level similar to that of untreated controls, probably as a result of decreasing insult.

The epithelial remnants of Malassez existing throughout the periodontal ligament showed intense expression of IL-1 β . Yamamoto et al.³⁸ and Tonetti³⁹ have demonstrated that oral keratinocytes produced IL-6, GM-CSF, and IL-8 apart from IL-1 β and TNF- α . Moreover, TNF- α induced dose-dependent expression of IL-8 in cultured gingival keratinocytes.³⁹ IL-1 β and TNF- α also stimulated IL-6 production and bone resorption synergistically.⁴⁰ IL1 injection into the rat gingiva increased the production of prostaglandin.⁴¹ IL-1 α and TNF- α were expressed in the bone and periodontal ligament (PDL) along roots of the orthodontically moved molars and in the gingiva.⁴² There is an increased expression of PDGF and TGF-beta in laser irradiated gingiva.

There appears to be a similarity in the expression of pro-inflammatory cytokines in the rat periodontium and the human periodontium. This has made the rat model as the closest one to be extrapolated to humans.

Suitability of a Rat Gingival Model in Extrapolating Results to Humans

Animal models are often considered superior to *in vitro* studies and are an important and essential link between hypotheses and its relevance to human patients. Animals have been the standard testing ground for drugs being developed or tested for safety and effect before entering any clinical trials. The use of healthy animals for drug assessment has many advantages. They are standardized and accepted by regulatory authorities. Inter-laboratory results can be compared and new results can be better interpreted as there is more data on such animal models.⁴³

The animal model suitable for drug testing should be one that resembles human inflammatory response. Rodent models have several useful features for investigating molecular mechanisms involved in the pathogenesis of inflammatory conditions. Indeed, there is considerable background information on mouse and rat immune systems and a wide range of immunologic and cellular reagents are available. Many of the same series of inflammatory events occur in the rat periodontium as in the non-human primate. In a rat periodontitis model, loss of attachment and bone loss occurred predictably in a 7 day period,^{44,47} although investigators have conducted experiments over much longer periods of time.⁴⁸⁻⁵¹

Like human periodontitis the destructive phase of experimental periodontitis in rats is associated with a host response as shown by the formation of an inflammatory infiltrate in the gingiva prior to bone resorption.⁵² When the host response was diminished by inducing endotoxin tolerance, bone loss was decreased.⁵³ Rats treated with prostaglandin inhibitors demonstrated a decreased inflammatory response. The COX-1/COX-2 inhibitor, indomethacin, reduced gingival inflammation, osteoclast formation and bone loss in a rat periodontitis model.³⁵ The COX-2 inhibitor, meloxicam, has a similar effect.³⁵ Low dose doxycycline, which inhibits MMP activity, also reduced ligature enhanced alveolar bone loss.³⁴

Rats are often used because periodontal anatomy in the molar region shares some similarities with that of humans. Furthermore, rats are easy to handle and can be obtained with different genomes and microbial status. Rats are inexpensive in many ways including procurement and its maintenance. As gingival inflammation is a typical inflammation and similar in occurrence in humans, the use of a rat gingival model to evaluate drugs on their anti-inflammatory potential could lead to a simple and exciting era of drug testing.⁵⁴

Various forms of models which can be considered are:

1. Normal gingiva
2. Traumatized gingiva (for acute inflammation)
3. Periodontitis model (for chronic inflammation)

Other Popular periodontal animal models

Two other commonly used animal models in periodontal research are the beagle dog model with natural periodontal disease and the non-human primate with ligature-induced attachment loss. The immunological characteristics of periodontal disease in the canine and non-human primate are similar to each other and to the human⁵⁵. The sequence of events in gingival inflammation in dogs begins with the formation of supragingival plaque. Plaque by-products enter the gingival tissues by diffusion. The gingiva responds to this insult by several host response mechanisms leading to a gingival sulcus.⁵⁶ Appearance of periodontitis generally requires between 1 and 3 years of experimental monitoring.⁵⁷ Several studies⁵⁸ have been performed assessing the effects of plaque-induced gingivitis and ligature-induced periodontitis⁵⁹ in *Macaca fascicularis* (monkeys). The progression of gingivitis to periodontitis has been closely followed in a temporal manner in the non-human primate utilizing the ligature-induced periodontitis model. Within 1 day following placement of ligatures for induction of periodontal attachment loss in monkeys, the marginal gingiva became acutely inflamed with ulceration of the junctional epithelium.

Though there is more justification on the use of higher animals, they have some serious issues which can hamper research. It is becoming increasingly difficult to acquire beagle dogs with natural periodontal disease.⁶⁰ Dogs in many breeding colonies currently receive a biannual oral hygiene regimen which slows the development of advanced periodontal disease, thus limiting their appropriateness for use in regenerative studies. The cost of using monkeys and dogs for research are considerably greater than small animals. The purchase of non-human primates can be made more economical, however, when utilizing animals that have been used

for other experiments, (e.g., following studies of the effects of microbial flora on disease progression or drug safety). However, if this option is utilized one must first determine the residual effects of previously administered agents which may affect bone and connective tissue metabolism or wound healing in general.

Limitations of an anti-inflammatory drug testing rat gingival model

Though it appears that the 'rat' is potentially the most suitable candidate for preclinical drug testing, a few important issues need to be addressed. The predictive validity of animal models is compromised by interspecific differences with humans, subject sample homogeneity and imperfect outcome measures. The most obvious problems stem from differences between species.⁶¹ As a result, animal models never fully recapitulate the human disorder.⁶² Disease symptoms, courses, outcomes, and the effects of various interventions vary radically across species. Interspecific variations in lifespan lead to time-related problems for animal models. Animals used to model human disease typically have much shorter lifespans than humans. The shorter lifespan may prevent an animal model from following the human disease trajectory. Disease characteristics of particular interest must occur within the shorter lifespan of the animal being tested.⁶¹

Animal studies typically use young, healthy populations of animals that are homogeneous for sex and age. This is done in order to avoid confounding of results by physiological remodeling processes.¹³ Such optimal demographics may decrease predictive validity, as these study populations are often a poor match for the heterogeneity of human patients for whom the interventions are being developed.⁶¹

It also seems probable that rat strains may differ with respect to susceptibility to periodontitis, but no experimental data are available in this area.¹³

Experiments are rarely extended beyond 100 days.^{18,19,29} Hence the chronic and unpredictable

nature of a chronic infection might not be truly replicated.⁵⁴ The small size of the animal and therefore the amount of tissue for analysis will require a large number of rats.

Conventional animal models for topical anti-inflammation drug testing

There are various animal models of inflammation which has been successfully used to test the action of topical drugs.⁶³ Soon after the second world war, a salt of aminophenazone, 'phenylbutazone' was found to have potent anti-inflammatory effects. A pharmacologist at Geigy, Gerhard Wilhelmi, developed novel models of inflammation. Phenylbutazone turned out to be particularly active in reducing erythema elicited in the depilated skin of guinea pigs following irradiation with ultraviolet (UV) light. This was probably an early model to study skin inflammation but the drug was administered systemically than topically. This was also the first model of inflammation used to define the activity of what we now call nonsteroidal anti-inflammatory drugs (NSAIDs) including the topical formulations.⁶⁴ The other models are also mainly based on skin inflammation. Chronic Skin Inflammation Model in mice is a chronic persistent skin inflammation in the ears of CD-1 mice induced by the repeated treatment of phorbol ester using the procedure of Stanley *et al.* (1991).⁶⁵ Ear punch biopsies were removed, weighed for assessment of edema, and then snap-frozen for later analysis of various inflammatory markers. The skin inflammation in this model was persistent and had been useful in assessing whether topically applied compounds were able to resolve an existing inflammatory lesion.⁶⁵

UVB-Induced Erythema in Hairless Guinea Pigs

Hairless guinea pigs are exposed on the masked flanks to UVB radiation (350 mJ) emitted from fluorescent tubes.⁶⁴ Topical agents are applied to assess the effect on the intensity of erythema.⁶⁶ A novel model of dermatitis was reported in a recent work by Yamamoto where 100 mg Dfb ointment was topically applied on upper

shaven back of NCr Nga mice. The established skin lesion in this model lasted for at least 2 weeks without further topical application of allergen and was suitable for testing topical drugs. This is very convenient for evaluation of the efficacy of topically applied drugs, because there would be no question about the possibility for an interaction between the drug and the antigen.

Irritant contact dermatitis in mice and rats

Both ears of Wistar rats are topically treated with (testing) drugs that are dissolved in croton oil solution. After 24 h, animals are killed and edema is determined by measuring ear weight or 10 mm diameter ear punch biopsy weight.⁶⁷ Parameters for neutrophil infiltration, elastase activities are analyzed in ear homogenate.

Allergic contact dermatitis in mice and rats

NMRI mice are sensitized in the skin of the flank with 25 mL of 0.5% dinitrofluorobenzene (DNFB) in ethanol at days 0 and 1.⁶⁷ On day 5; mice are challenged by topical application of 20 mL of 0.3% DNFB as described by Zügel et al.⁶⁸ Wistar rats are sensitized with 75 mL of 0.5% DNFB at day 0. On day 5, rats are challenged by topical application of 40 mL 0.4% DNFB. Test compounds are topically co-applied with the hapten challenge. After 24 h, animals are killed to determine ear weight and elastase activity from ear homogenates as parameters for edema and neutrophil infiltration.

Carrageenan-induced paw edema

In the Wistar Rats, foot pad thickness is measured after inducing local inflammation by injecting 1% carrageenan (w/v) into the plantar surface of a hind paw.⁶⁹ Topical agents are applied to find any change in thickness as a sign of anti-inflammation.

Conclusion

Rat gingival tissues are suitable for topical drug testing acting on inflammation. As there is a constant presence of inflammation in gingiva, it can be used to find if the drug

is sensitive and potent enough to reduce inflammation. Acute inflammation can be induced by traumatizing the gingiva and evaluating the drug response on it. A chronic inflammation can also be induced in rats to study the effect of drugs on chronic inflammation. As there is no need of external chemicals to induce inflammation there is no fear of interaction with the testing drugs. A rat gingival model will be cheaper compared to other inflammatory models but more research is required to confirm whether the proposed use of rat gingival tissues for drug testing is valid and feasible. Future research should be done to compare the rat and human gingiva with respect to drug kinetics, inflammatory response and wound healing.

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Table I: Comparison of cytokines present in healthy gingiva of rats and humans.

Cytokines	Human gingiva	Rat Gingiva
Epidermal growth factor	Present	Present
Platelet derived growth factor	Present	Present
Transforming growth factor beta 1	Present	Present
Interleukin 1beta	Present	Present
Interleukin 6	Present	Present
Interleukin 8	Present	Present
Interleukin 13	Present	Absent

Table II: Comparison of cytokines present in inflamed gingiva of rats and humans.

Cytokines	Human gingiva	Rat Gingiva
Platelet derived growth factor	Present	Present
Transforming growth factor beta 1	Present	Present
Interleukin 1beta	Present	Present
Interleukin 6	Present	Present
Interleukin 8	Present	Present
Tumour Necrosis factor- alpha	Present	Present

Table III: Comparison of various animal models for topical drug testing.

	Rat gingival	Chronic Skin Inflammation Model in Mice	UVB-Induced Erythema in Hairless Guinea Pigs.	Dfb ointment mice	Irritant contact dermatitis in mice and rats	Carrageenan-induced paw edema. In the Wistar Rats
Interaction with testing drugs	×	✓	×	×	✓	×
Inflammatory markers	✓	✓	×	×	✓	×
Chronicity	✓	✓	×	×	✓	×
Ease of inducing inflammation	✓	×	×	×	×	×