Published in final edited form as:

Bone. 2020 January ; 130: 115141. doi:10.1016/j.bone.2019.115141.

Anti-Vascular Endothelial Growth Factor Antibody Monotherapy Causes Destructive Advanced Periodontitis in Rice Rats (*Oryzomys palustris*)

J.G. Messer¹, E.J. Castillo¹, A.M. Abraham¹, J.M. Jiron¹, R. Israel¹, J.F. Yarrow^{2,3}, S. Thomas¹, M.C. Reynolds², R.D. Wnek², M. Jorgensen⁴, N. Wanionok¹, C. Van Poznak⁵, I. Bhattacharyya⁶, D.B. Kimmel¹, J.I. Aguirre¹

¹Department of Physiological Sciences, University of Florida (UF), Gainesville, FL.

²VA Medical Center, Research Service, VA Medical Center, Gainesville, FL.

³Division of Endocrinology, Diabetes, and Metabolism, University of Florida College of Medicine, Gainesville, FL.

⁴Department of Pediatrics, College of Medicine, UF.

⁵University of Michigan, Ann Arbor, Michigan.

⁶Department of Oral & Maxillofacial Diagnostic Sciences, College of Dentistry, UF.

Abstract

OBJECTIVE: Angiogenesis inhibitors (AgI) are commonly used in combination chemotherapy protocols to treat cancer, and have been linked to osteonecrosis of the jaw (ONJ). However, it is unknown if AgI therapy alone is sufficient to induce ONJ. We have previously established an ONJ model in rice rats with localized periodontitis that receive zoledronic acid (ZOL). The purpose of this study was to use this model to determine the role of anti-vascular endothelial growth factor A (anti-VEGF) antibody treatment of rice rats with localized maxillary periodontitis. We hypothesized that rice rats with localized maxillary periodontitis given anti-VEGF monotherapy will develop oral lesions that resemble ONJ, defined by exposed, necrotic alveolar bone.

METHODS: At age 4 wks, 45 male rice rats were randomized into three groups (n=15): 1) VEH (saline), 2) ZOL (80 µg/kg body weight, intravenously once monthly), and 3) anti-VEGF (5mg

Please address correspondence to: J. Ignacio Aguirre, D.V.M., Ph.D., DACLAM, Department of Physiological Sciences, Box 100144, JHMHC, University of Florida, Gainesville, FL 32610, Telephone: (352) 294-4038, FAX: (352) 392-5145, aguirrej@ufl.edu.

Authors' roles:

Study design: JIA, DBK. Study conduct: JGM, JIA, AMA, MJJ, EJC. Data collection: JIA, AMA, JGM, DBK, RI, ST, MCR, RDW, MJ, NW, MJJ, EJC. Data analysis: JIA, JGM, DBK, JFY, IR, NW, MCR, RDW. Data interpretation: JIA, JGM, DBK, NB, CVP. Drafting manuscript: JIA, DBK, and JGM. Revising manuscript content: JIA, JGM, JFY, MJJ, EJC, CVP, NB, and DBK. Approving final version: JIA, EJC, AMA, MJJ, RI, JFY, NW, ST, MCR, RDW, MJ, CVP, NB, CVP, and DBK. JIA takes responsibility for the integrity of the data.

Conflict of Interest

The authors have no conflicts of interest. CVP's Institution receives research support from Bayer. CVP receives a small amount of salary support from the research support and royalties from UpToDate for writing for Bayer.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

B20-4.1.1/kg body weight, subcutaneously twice weekly). After 24 wks, rats were euthanized, jaws were excised and a high-resolution photograph of each quadrant was taken to assign a severity grade based on gross appearance. Jaws were then fixed, scanned by MicroCT, decalcified and sectioned for histopathologic and immunohistochemical analyses.

RESULTS: 40-80% of the rats in the three groups developed gross oral lesions. 50% of ZOL rats developed ONJ. In contrast, 80% of the anti-VEGF rats developed destructive advanced periodontitis that was characterized by extreme alveolar bone loss and fibrosis. Anti-VEGF rats never developed exposed, necrotic bone. Furthermore, only anti-VEGF rats developed mild to severe mandibular periodontitis. Compared to VEH rats, more T-cells were found in periodontal lesions of anti-VEGF rats and more cells of the monocyte lineage were found in ONJ lesions of ZOL rats.

CONCLUSIONS: Anti-VEGF monotherapy administered to a validated rodent model of ONJ caused a destructive advanced form of periodontitis that differed significantly from ONJ.

Keywords

anti-angiogenic; oncology; oral diseases; osteonecrosis; jaw

Introduction

Osteonecrosis of the jaw (ONJ) is a potentially severe side effect associated with the use of powerful anti-resorptive drugs, including nitrogen-containing bisphosphonates [e.g., zoledronic acid (ZOL)] [1-4], and receptor activator of nuclear factor κ B ligand (RANKL) antibodies (e.g. denosumab) in humans [5, 6]. More recently, the systemic administration of angiogenesis inhibitors (AgIs), including the anti-vascular endothelial growth factor A (VEGFA) antibody bevacizumab and receptor tyrosine kinase (RTK) inhibitors, has also been associated with ONJ [7-15]. ONJ is defined as exposed bone, or bone that can be probed through a fistula, in the maxillofacial region, that has persisted for more than eight weeks in patients with no history of radiation therapy to the jaws, no obvious metastatic disease in the jaws, and a history of powerful anti-resorptive and/or AgI therapies [14].

ONJ has an estimated prevalence of 2.1%, 3.8% and 5.2% in patients with breast cancer, prostate cancer, and multiple myeloma, respectively [16]. From the ONJ patient population perspective, 29.5%, 9.6%, and 31.2% have breast cancer, prostate cancer, and multiple myeloma, respectively [17]. Bevacizumab and RTKIs are AgIs approved by the USFDA for adjuvant use as chemotherapy in selected cancers (Supplemental Table 1). A recent review of ONJ related to non-powerful anti-resorptive medications identified bevacizumab in 36% and RTKIs in 33% of cases, respectively [15].

Though powerful anti-resorptive-associated ONJ has been extensively studied clinically and preclinically [1-6], only limited information is available on AgI-associated ONJ. Most evidence concerning an association between systemically administered AgIs and ONJ comes from case reports and case series [7-11], retrospective studies [12, 18, 19], and three placebo-controlled trials of oncology patients whose treatment regimen included bevacizumab [13]. All patients were previously or concurrently treated with powerful anti-

resorptives [12, 18-20], anti-neoplastic drugs [7-11, 13], or various combinations of the two [10, 12]. The use of concurrent medications with AgIs by patients with ONJ, particularly powerful anti-resorptives with their known relationship to ONJ, makes it difficult to understand the relationship of AgIs to ONJ from these studies alone.

Testing AgI monotherapy in animal models previously used to study powerful anti-resorptive-induced ONJ could provide important data about the relationship of AgIs to ONJ. Current evidence concerning the etiology of powerful anti-resorptive-induced ONJ indicates that a combination of the systemic medication and a permissive local oral risk factor is required. The most prominent oral risk factor for ONJ is recent dento-alveolar surgery (e.g. tooth extraction) [1, 4, 14, 21]. Oral infection/inflammation, including periodontitis and periapical infection has also been identified as an important risk factor for ONJ in humans [2-4, 22-24] and in different animal models [25-29]. Infection may even account for most tooth extraction-related ONJ, because the great majority of tooth extractions performed in adult humans involve teeth with active infection that originated from caries or periodontal disease [1, 2, 14, 17]. The role of infection/inflammation has been especially recognized in bisphosphonate-related ONJ, of which many more cases have been surveyed than for either denosumab-related ONJ and AgI-related ONJ [14, 30-35]. However, it should be noted that coinciding oral infections were reported for nearly half of ONJ patients taking denosumab [36], and periodontitis or periapical infection have also been linked to RANKL inhibitors-induced ONJ in rodents [37, 38]. Periodontitis and complications of oral infections have also been reported in patients taking AgI-containing combination therapies [39-44], and pre- or co-existing oral conditions were specifically observed in patients who received AgIs and later developed ONJ [7, 43, 45].

Although healing of tooth extraction sites as a local oral risk factor has been studied in mice given an anti-VEGFA antibody [46], no studies have investigated whether AgIs by themselves can promote the onset of ONJ in association with periodontitis as the local oral risk factor. For this purpose, we performed a preclinical experiment using AgI monotherapy in the rice rat (*Oryzomys palustris*) because this species is naturally susceptible to periodontitis [29, 47-49], and periodontitis-affected rice rats develop well-characterized bisphosphonate-induced ONJ when given clinically-relevant doses of ZOL [26, 50, 51] or clodronate [52]. An anti-VEGFA antibody was selected because bevacizumab is the single AgI most frequently involved in AgI-related human ONJ cases [15]. We hypothesize that rice rats with evolving periodontitis develop ONJ when simultaneously given anti-VEGFA monotherapy.

Materials and Methods

Animal Care and Management

Rice rats were generated in-house as previously [50]. Two to five rats were housed per cage (area: 143 in²) in pine shaving bedding and continuous access to food and water. Housing rooms were maintained at 20-26°C with humidity at 30-70% and a 12:12hr light:dark cycle (14:10hr cycle for breeders). Experimental protocols (#201708453 and #201708452) were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

Justification for using the Rice Rat Localized periodontitis Model

Rice rats develop two distinctive types of periodontitis with no local mechanical intervention: 1) a generalized form, which affects both jaws and is achieved by feeding a high sucrose-casein (HSC) diet [47-49]; and 2) a localized form, which most often affects the interdental space between the second and third maxillary molars, and is achieved by feeding rice rats standard (STD) rodent chow [29]. The defining feature of localized periodontitis is impacted material that directly injures soft tissues at the lingual aspect of the interdental space between the maxillary second-third molars, establishing a localized chronic inflammatory response. This localized periodontitis model is relevant to humans because food impaction around teeth, fixed partial prostheses, dental implants, or embrasures in humans [53], is an important risk factor for localized periodontitis in humans [54]. The rice rat localized periodontitis model has three key advantages over the rice rat generalized periodontitis model for this study: a) localized periodontitis lesions consistently occur in untreated rats at a specific location (lingual aspect of the second-third maxillary molar interdental space) with a high prevalence (up to 80% at age 28 wks) [29], making them relatively common in a site with potentially good visual access in vivo; b) rice rats with localized periodontitis that receive clinically-relevant doses of ZOL, develop a high prevalence of oral lesions that grossly and histologically resemble human ONJ at the anatomical location where localized periodontitis lesions first appear [50]; and c) the prevalence of ONJ is significantly higher in the localized periodontitis/ZOL/ONJ model than the generalized periodontitis/ONJ model [26, 50, 51]. These features may make it practical to do longitudinal observation of the initiation/progression of lesions by repeated clinical examination of anesthetized rats.

Study Design

Forty five clinically healthy, male rice rats (age 4 wks., body weight 30g and body condition score 3.0) were randomized into three groups (n=15): 1) VEH (0.9% saline vehicle control) 2) ZOL (ONJ positive control), and 3) anti-VEGFA antibody B20-4.1.1 [55, 56].

All rats were fed STD diet (Envigo Teklad LM-485 (irradiated 7912) Rodent Diet; Tampa, FL USA) and received water ad libitum. Twice weekly injections of anti-VEGFA, monthly injections of ZOL, and oral exams started at age 4 wks and ended after 24 wks treatment. Rats in the anti-VEGFA group received subcutaneous injections of anti-VEGFA monoclonal antibody (B20-4.1.1; 5 mg/kg body weight twice weekly). Bevacizumab only inhibits human VEGFA. B20-4.1.1, a rodent surrogate of bevacizumab that inhibits both human and rodent VEGFA, is specifically designed for safe administration at a dose of 5 mg/kg body weight twice weekly in rodent studies [55] that last up to 30 weeks [56]. This dosing regimen produces similar minimum serum concentrations of anti-VEGFA activity to those seen in patients taking bevacizumab as adjuvant cancer chemotherapy [55, 57-61], thereby creating conditions in nontumor tissues of rodents that parallel what occurs in non-tumor tissues of humans receiving bevacizumab. B20-4.1.1 was provided by Genentech (South San Francisco, CA, USA). B20-4.1.1 (17.26 mg/mL) was diluted to a working stock of 0.35 mg/mL in saline (pH 7.2) on the day of injection. Rats in the ZOL group received an oncology equivalent dose of ZOL (80 µg/kg body weight intravenously every 4 wks.) [26,

50]. ZOL was dissolved in sterile normal saline (pH 7.2, 0.2 mg/ml), and injected at 0.4 mL/100g body weight into the tail vein of each rat placed in a rodent restrainer. ZOL was provided by Novartis Pharma AG (Basel, Switzerland). Rats in the VEH group were injected with either subcutaneous or intravenous saline on the appropriate days. Rats exhibiting body weight loss and/or body condition score deterioration were monitored daily and offered gel diet and supplemental fluids.

In vivo oral exams

Oral exams were performed bi-weekly to assess the prevalence and severity of maxillary lesions, because of the excellent visual access to the lingual aspect of maxillary molar interdental spaces and the high prevalence of localized periodontitis in the maxillary interdental space between the second and third molars in this model [29, 50]. Previous studies indicated that rice rats at age 4 wks. have no gross or histopathological evidence of localized periodontitis in the interdental space between the second and third maxillary molars [29, 50]. Therefore, oral exams were started at age 6 wks. in rats anesthetized by isoflurane at concentrations of 4% v/v and 2-3% v/v for induction and maintenance, respectively [62]. Rats were presented in random order to the examiners (JGM, EJC) who were cross-calibrated and blinded to treatment group. Briefly, anesthetized rats were placed in dorsal recumbency on a heated surgical platform (Kent Scientific Corporation; Torrington, CT) (Supplemental Figure 1). After placement of an anesthesia nose cone, the mouth was gently held open with small blunt forceps positioned behind the incisors, and each cheek was sequentially retracted using a second pair of closed bent forceps. Maxillae were examined with the aid of dental loupes (3.5x magnification) fitted with a LED headlight. Each maxillary quadrant was assigned a gross quadrant grade (GQG) that indicated lesion severity (Table 1) [29, 50, 51].

Euthanasia and Tissue Collection

After 24 weeks, rats were euthanized by CO₂ inhalation followed by cervical dislocation. Cardiac puncture was performed, yielding ~1-1.5 mL of whole blood. Blood was allowed to clot at room temperature and centrifuged at 3000 rpm for 10 minutes. Serum was collected and stored at -20°C. Maxillae and mandibles were excised and trimmed, leaving gingiva and oral mucosa intact [50]. High resolution photographs from the medial aspect of each hemi-mandible and palatal aspect of each hemi-maxillae were taken with a digital camera (Canon EOS 6D; Tokyo, Japan) attached to a macro lens (Canon EF 100 mm 1:2.8; Tokyo, Japan). Right femur, heart, kidney, liver, lung, spleen, and adipose tissue were also excised. Organs and jaw quadrants were fixed at 4°C in 4% paraformaldehyde for 48 hours, and then transferred to 70% ethanol. Microcomputed tomography (MicroCT) scans of selected jaw quadrants were obtained. Histopathologic and immunohistochemistry analyses of selected jaw quadrants were completed. Femur length was measured with digital calipers.

Measurement of Serum VEGF

Serum VEGF concentration was assessed in all groups (n=13-15) using a rat specific VEGF ELISA kit (ab100786, Abcam, Inc., Cambridge, UK), following manufacturer's instructions.

Gross Quadrant Grade (GQG) Screening

To assess severity of gross lesions and guide selection of jaw quadrants for MicroCT and histopathologic examination, a scoring system (from 0-4) was applied (Table 1) [29, 50]. *In vivo* GQGs were assigned only to the maxillary quadrants during oral exams. *Ex vivo* GQGs were assigned to both maxillary and mandibular quadrants by examining the high resolution photographs. All photographs were presented for GQG scoring in randomized order to treatment-blinded investigators (JGM; JIA; DBK). The GQG for a rat represents the highest GQG found in any jaw quadrant of the rat during any examination.

MicroCT

MicroCT scanning was performed on a subset of 28 maxillary quadrants as follows: 1) anti-VEGFA rats with GQG 0 (n=4) or GQG 4 (n=6), 2) VEH rats with GQG 0 (n=6) or GQG 1-2 (n=6), and 3) ZOL rats with GQG 2-4 (n=6). Images were acquired using the following parameters: 80 kVP/120 μ A, 0.5mm aluminum filter, 2 k camera resolution, 10 μ m voxel size, 0.5° rotation step, and 360° tomographic rotation. Cross-sectional images were reconstructed using a filtered back-projection algorithm (Bruker Skyscan, NRecon, Kontich, Belgium). All 2D images were aligned identically in the mesiodistal plane with all molars simultaneously visible.

Histopathologic Analysis of Jaws

Specimen Selection, Preparation, and Examination of Jaw Quadrant Sections.

—Previous ONJ studies using the rice rat model found that jaw quadrants with GQG 0-1 never had histopathologic ONJ lesions. In contrast, ~15%, ~40%, and ~50% of quadrants with GQGs 2, 3, and 4, respectively, had ONJ on histopathologic examination [50]. Thus, all quadrants with GQG 2 underwent histopathologic examination. At least one maxillary and one mandibular quadrant from each rat was examined histologically, including rats whose jaw quadrants had only GQG<2. For rats with two or more quadrants with GQG 2, the highest GQG from an arch was initially analyzed. If this quadrant was free of histopathologic ONJ, we evaluated the consecutive quadrant that followed in severity level as needed, until the rat was either diagnosed with ONJ or confirmed ONJ-free. A rat with ONJ in at least one quadrant was considered positive for ONJ. Jaw quadrants were decalcified, sectioned, and stained as before [50]. Serial sections were used to diagnose ONJ, assign PD Score, quantify alveolar bone loss, and analyze immune cell markers and osteoclasts as previously described [50].

Histopathologic Definition of ONJ Lesions and Quantification of Necrotic Bone.

—We utilized histopathology to establish a definitive diagnosis of ONJ for individual rats (Table 2). To establish a diagnosis of ONJ, a quadrant was required to have both exposed and necrotic bone in at least one level [63]. Exposed bone was identified by the absence of overlying gingival epithelium, lamina propria, and periodontal ligament [26, 50, 51] (Table 2). Necrotic bone was identified using: 1) a pattern recognition approach [64-66], and 2) specific criteria used in previous pre-clinical studies of ONJ that require bone matrix containing 10 adjacent lacunae that were either empty or contained pyknotic osteocyte nuclei or cellular debris [67, 68]. Two observers (JGM, JIA) surveyed all levels

independently. When their diagnoses differed, JGM and JIA reached agreement by reviewing slides together. A rat with at least one ONJ positive quadrant was considered positive for ONJ.

The total number of osteocyte lacunae and the number of empty lacunae, indicated by absence of osteocyte nuclei or presence of pyknotic osteocyte nuclei or cellular debris, was counted [50, 51]. The number of live osteocytes examined is the total number of osteocyte lacunae minus the number of empty lacunae. Data collection was performed at 200× magnification in sections within a 0.15–0.25 mm² ROI that contained localized periodontitis showing periodontal inflammation and tissue destruction in localized periodontal lesions. The total number of lacunae per bone area (density of osteocyte lacunae, #/mm²), number of empty lacunae per bone area (#/mm²), and percentage of empty lacunae were calculated.

Histopathologic Assessment of Periodontitis

PD Score.: Histopathologic periodontitis severity was determined in maxillae and mandibles based on a previous PD Score system [26, 51] which was modified to capture unique features of localized periodontitis (Table 2). The PD Score for a rat is the greatest PD Score found in any of its quadrants. Quadrants with PD Score 1 were considered positive for gingivitis, while quadrants with PD Score 2 were considered positive for periodontitis. H&E-stained sections were analyzed by one observer (JGM), blinded to both GQG and treatment, using an Olympus BX43 microscope (Olympus Center Valley, PA, USA).

Alveolar Bone Loss.: Alveolar bone loss was determined by measuring alveolar bone height. It is defined as the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest using sections oriented in the mesio-distal plane [49, 69]. The larger the CEJ-alveolar bone crest distance, the greater the alveolar bone loss. Alveolar bone loss was measured separately at the interdental spaces between the first and second molars and the second and third molars [49, 69] in the maxillary and mandibular quadrants with the highest GQG from each rat. In rats with only GQG 0 quadrants, the left quadrants were used. Measurements were made by a blinded observer (JGM) on tissues randomized by GQG and treatment. Interdental spaces showing a complete absence of alveolar bone were assigned a default value of 1500 µm [49, 69].

Histopathologic evaluation of organs and tissues.

At necropsy, tissue samples from heart, lungs, liver, kidney, spleen, and adipose tissue were collected, fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin blocks were sectioned using Accu-Cut SRM 200 Sakura microtomes (Sakura Finetek Europe B.V, Zoeterwoude, NL). Sections were stained with H&E, coverslipped, and examined by light microscopy.

Immunohistochemical Analysis

To further characterize the response of periodontal tissues to treatments, we performed a quantitative analysis of cells known to play an important role in periodontitis and angiogenesis using immunohistochemistry as previously described [51, 70]. Maxillae from VEH rats with no periodontitis (PD Score 0 or 1) (n=5-7) or periodontitis (PD Score 2-3)

(n=5), ZOL rats with no periodontitis (n= 5) or with ONJ (n=4-6), and anti-VEGFA rats with periodontitis (PD Score 4) (n=5-9) were analyzed. The following primary antibodies were used: 1) rabbit polyclonal anti-TRAP, a specific protease present in osteoclast lysosomes, (ab192780, Abcam, Cambridge, United Kingdom, 1.6 µg/ml); 2) rabbit polyclonal anti-CD3, present in T cell co-receptor for CD4 and CD8 positive cells, (A0452, Dako, Carpinteria, CA, 1 µg/ml); 3) mouse monoclonal anti-CD68, highly expressed by cells in the monocyte lineage including macrophages, pericytes, osteoclasts, etc., (ab31630, Abcam, Cambridge, MA; 3.3µg/ml); and 4) rabbit anti-CD31 for blood vessel endothelial cells (ab28364, Abcam, Cambridge, MA; 20 µg/ml). Longitudinal sections through the maxillary interdental spaces were selected for analysis and a region of interest (ROI) was designated. The ROI began 300 µm mesial to the first molar mesial root and ended 300 µm distal to the third molar distal root. The ROI was bounded by the CEJ on the coronal aspect and the inferior border of the mandible on the apical aspect. For CD31 expression, we reported the data specifically collected at the maxillary interdental area between the second and third molars, which is the area primarily affected in this model. Longitudinal sections of mandibles were also used to identify blood vessels with the anti-CD31 antibody. Data from the mandibles were collected at the interdental area between the first and second molars, a location that is less affected in the localized periodontitis model and was used as a comparative region. Alveolar bone perimeter (B.Pm) was measured and TRAP⁺ cells (#) on alveolar bone surfaces were counted. Because CD31 is also expressed on plasma cells, macrophages, megakaryocytes, etc., only the CD31⁺ blood vessels were counted, excluding molar roots and marrow cavity. Cell number, blood vessel number, tissue area, and bone perimeter were measured using Osteomeasure software (Osteometrics Corporation; Decatur, GA USA). The number of TRAP⁺ cells per millimeter of B.Pm was calculated (#/mm). The numbers of CD3⁺, CD68⁺ cells, and CD31⁺ blood vessels were expressed per square millimeter of tissue (#/mm²).

Statistics

Prevalence of rats with gross lesions (GQG = 0.5) based on oral exams was evaluated with a Chi-square test. Prevalence of rats with gross lesions (GQG > 0) based on *ex vivo* analysis and prevalence of rats with histopathologic lesions were assessed with a Fisher Exact Test. Oral exam GQG and body weight were assessed with two-way ANOVA on repeated measures with treatment and treatment duration as the main effects. Alveolar bone loss was assessed with two-way ANOVA with periodontal status of no (PD Score 0-1) or yes (PD Score 2-4) and treatment as the main effects. Significant differences within each time point were determined by Holm-Sidak post hoc analysis. Immunohistochemistry, osteocyte lacunae, alveolar bone loss, and femoral length data were assessed with one-way ANOVA with Holm-Sidak post hoc analysis. Data are expressed as Mean±SD, except when otherwise indicated.

Results

General Observations (Body Weight, Femoral Length, and Histopathologic Organ Evaluation)

All rats completed the study, except one ZOL rat that died during intravenous injection at age 8 wks. Body weight did not differ at any time point among groups, except for anti-VEGFA rats that had lower body weight compared to VEH rats at wks. 18-24 ($p < 0.05$) and ZOL rats at wks. 20-24 wks. ($p < 0.05$) (Supplemental Figure 2A). Femoral length did not differ among groups (Supplemental Figure 2B). Histopathologic evaluation of organs disclosed no significant abnormalities.

Serum VEGF Concentration

VEH and ZOL treated rats had non-detectable levels of serum VEGF. In contrast, anti-VEGFA rats showed significantly higher serum VEGF levels compared to VEH ($p < 0.05$) and ZOL ($p < 0.05$) rats (Supplemental Figure 2C).

Gross Analysis of Oral Lesions

In vivo exams.—Lesions tended to be more prevalent in anti-VEGFA rats compared to VEH rats ($p = 0.06$) during wks. 17-23 (Figure 1A). Lesion severity in VEH rats was relatively stable throughout study. However, there were significant effects of duration ($p < 0.001$) and treatment ($p < 0.034$) on lesion severity (Figure 1B). Severity worsened significantly in anti-VEGFA rats ($p < 0.036$), but not in ZOL rats ($p = 0.19$) (Figure 1B). Anti-VEGFA caused significantly more severe lesions compared to VEH at wk. 13 and wks. 17-23 ($p < 0.05$) (Figure 1B). Though oral exams proved useful for early detection and longitudinal observation of lesions, some mouth regions (e.g. buccal aspect of maxillary molars, all aspects of mandibles) were difficult to assess due to poor visual access.

Ex vivo exams.—The ex vivo exam revealed the same tendency for a higher prevalence of lesions in anti-VEGFA compared to VEH rats ($p = 0.06$) as the final oral exam (Figure 1C). Maxillary GQG4 lesions were identified only in the ZOL and anti-VEGF groups. Gross lesions were more prevalent in ZOL and anti-VEGFA rats compared to VEH rats ($p = 0.017$; $p = 0.001$, respectively), and gross lesions were more prevalent in anti-VEGFA compared to ZOL rats ($p = 0.025$) (Figure 1C).

Representative maxillary photographs from rats with different grades of lesions are shown (Figure 1D). Mild localized periodontitis (GQG 2) was found around the interdental space between the maxillary second and third molars in the VEH and ZOL groups. These features included mucosal recession, impacted materials, and tooth migration. GQG3 (moderate) and GQG4 (severe) lesions in ZOL and anti-VEGFA rats were open wounds that often extended beyond the interdental space between the maxillary second and third molars onto the palate and buccal mucosa, involving tissues adjacent to the first molar and distal to the third molar (Figure 1D). The prevalence of rats with bilateral maxillary lesions and rats with mandibular lesions was assessed (Supplemental Figure 2D). Though significantly more anti-VEGFA rats had bilateral maxillary lesions compared to VEH ($p = 0.008$) or ZOL ($p = 0.025$) rats, there was no difference between ZOL and VEH groups. There also tended to be more gross

mandibular lesions in anti-VEGFA rats compared to VEH ($p = 0.08$) and ZOL ($p = 0.08$) rats (Figure 1E). The percentage of maxillary, mandibular, and total quadrants with lesions was significantly greater in the anti-VEGFA group compared to either the VEH or ZOL group (Table 3).

Histopathologic Analysis of Oral Lesions

Prevalence of ONJ: Evaluation of exposed necrotic bone.—Maxillae of ZOL rats had areas of exposed alveolar bone that contained multiple empty osteocyte lacunae (Figures 2A-C), a histopathologic hallmark of ONJ [63]. Exposed dead bone could be seen in both the palate (Figure 2A) and the interdental space between the maxillary second and third molars (Figures 2B-C). The exposed bone and adjacent bone areas generally contained enlarged empty osteocyte lacunae (Figures 2a-c). In contrast, although the prevalence and severity of gross maxillary lesions in anti-VEGFA rats was greater than in ZOL rats, exposed necrotic bone was never found (Figures 2D-F). Twelve of the fifteen anti-VEGFA rats displayed an extremely destructive advanced form of periodontitis, in which little alveolar bone remained. All alveolar bone that remained in the anti-VEGFA rats with destructive advanced periodontitis was vital (Figure 2d-f). The percentage of empty osteocyte lacunae (Figure 2G) and empty osteocyte lacunae/bone area (Figure 2H) were significantly higher in ZOL rats compared to VEH ($p < 0.05$) and anti-VEGFA ($p < 0.05$) rats with periodontitis. VEH and anti-VEGFA rats did not differ for these features. Seven ZOL rats, but no anti-VEGFA or VEH rats, had ONJ. Thus, the prevalence of ONJ was significantly higher in ZOL rats (50%) compared to VEH (0%; $p < 0.002$) and anti-VEGFA rats (0%; $p < 0.002$) (Figure 2I).

PD Score of Maxillary Lesions and other histopathologic features.—93% of VEH rats developed oral lesions that ranged from gingivitis to mild-moderate periodontitis (Figure 2I). In ZOL rats that did not develop ONJ, lesions ranged from gingivitis to mild periodontitis. 80% of anti-VEGFA rats had destructive advanced periodontal lesions (Figure 2I), with a prevalence significantly higher than VEH rats ($p = 0.025$). Histopathological features of the destructive advanced periodontal lesions in anti-VEGF rats are fully described in Figures 2J-L. Necrotic exposed bone and other histopathologic features in ZOL rats are described in Figures 2M-O.

Maxillary Alveolar Bone Loss.—Since anti-VEGFA rats developed destructive advanced periodontitis but not ONJ, we further characterized periodontitis severity by measuring alveolar bone loss (Figure 3). Anti-VEGFA rats had more alveolar bone loss compared to either VEH or ZOL rats. At the interdental space between the maxillary second and third molars, the site where localized periodontitis usually initiates in this model, alveolar bone loss was greater in VEH rats with periodontitis, anti-VEGFA rats with periodontitis, and ZOL rats with ONJ, compared to the no periodontitis rats in each respective treatment group [VEH ($p < 0.001$); ZOL ($p = 0.008$); anti-VEGFA ($p < 0.001$)] (Figure 3A). Similar data were obtained when measurements were taken adjacent to the palatal bone next to the alveolar ridge (data not shown). Alveolar bone loss at the interdental space between the second and third molars was also significantly greater in anti-VEGFA rats with periodontitis compared to VEH rats with periodontitis and ZOL rats with ONJ

(Figures 3A and 3C-F). At the interdental space between the first and second molars, where localized periodontitis rarely initiates, alveolar bone loss was significantly greater in anti-VEGFA rats with periodontitis compared to all other groups (Figures 3B-F).

Prevalence of Histopathologic Lesions and Periodontitis Severity in the

Mandible.—Because we observed gross lesions in the mandibles of anti-VEGFA rats (Figure 1E), PD Scores were assigned to all quadrants (Table 3). While VEH and ZOL rats had mostly gingivitis (PD Score 1), anti-VEGFA rats had significantly higher PD Scores compared to both VEH ($p = 0.007$) and ZOL ($p = 0.022$) rats (Figure 3G). Alveolar bone loss was significantly greater at both molar interdental spaces in anti-VEGFA compared to VEH or ZOL rats (Figure 3H). There was no ONJ in any mandible. Mandibular periodontal lesions in anti-VEGFA rats had more extensive junctional epithelial migration, periodontal ligament disruption, and alveolar bone resorption (Figures 3K-L) compared to VEH or ZOL rats (Figures 3I-J).

Maxillary Bone Analysis (MicroCT).—Rats with localized periodontitis had greater alveolar bone loss, particularly at the interdental space between the second and third molars, than rats with no periodontitis (Figures 4A-B). ZOL rats with ONJ had more alveolar bone loss at the interdental space between the second and third molars than VEH rats with periodontitis (Figure 4C). Additionally, the surrounding alveolar bone of ZOL rats with ONJ had a pitted, honeycomb-like appearance, as previously described [26, 51] (Figure 4C). In contrast, anti-VEG rats showed more extreme alveolar bone loss at the interdental space between the second and third molars compared to VEH rats with periodontitis and ZOL rats with ONJ, which frequently involved the interdental space between the first and second molars (Figure 4E).

Localization of Cells Involved in Inflammation, Immune Response and Angiogenesis in Periodontal Tissues

Osteoclasts: TRAP⁺ cells were found at the alveolar bone surfaces in VEH/no periodontitis rats (Figure 5A), VEH/periodontitis rats (Figure 5B), ZOL/no periodontitis rats (Figure 5C), ZOL/ONJ rats (Figure 5D), and anti-VEGFA/periodontitis rats (Figure 5E).

There were significantly more TRAP⁺ cells in VEH/periodontitis rats (Figures 5B and 5F), ZOL/ONJ rats (Figure 5D and 5F), and anti-VEGFA/periodontitis rats (Figure 5E and 5F), compared to VEH/no periodontitis rats ($p < 0.05$) (Figures 5A and 5F), and ZOL/no periodontitis rats ($p < 0.05$) (Figures 5C and 5F). There were no differences among the treatment groups in rats with periodontitis.

T-cells: CD3⁺ cells were present predominantly within the gingival epithelium and lamina propria of VEH/no periodontitis rats (Figure 5G), VEH/periodontitis rats (Figure 5H), and ZOL/no periodontitis rats (Figure 5I). In ZOL/ONJ rats, CD3⁺ cells were present in both the gingival epithelium, lamina propria, and the fibrous tissue (FT) that typically surrounds the necrotic alveolar bone (Figure 5J). Anti-VEGFA rats with destructive advanced periodontitis also had CD3⁺ cells in the gingival epithelium and lamina propria, and had abundant CD3⁺ cells in the underlying fibrous tissue (Figure 5K). Consistent with their destructive advanced

periodontitis, there were significantly more CD3⁺ cells in the maxillae of anti-VEGF/periodontitis rats compared to VEH/no periodontitis and VEH/periodontitis rats, and ZOL/no periodontitis rats ($p < 0.05$) (Figure 5L). There tended to be more CD3⁺ cells in anti-VEGF/periodontitis rats compared to ZOL/ONJ rats (Figure 5L).

Cells of the Monocyte Lineage: CD68⁺ cells were found in the lamina propria, periodontal ligament, blood vessels (BVs), at alveolar bone surfaces, and occasionally, within the gingival epithelium of periodontal tissues from all groups (Figures 5M-Q). The microanatomical location of immunolabeled cells was consistent with the known expression of CD68 in macrophages, pericytes, and osteoclasts. There were significantly more CD68⁺ cells in ZOL/ONJ rats compared to VEH/no periodontitis, VEH/periodontitis, and ZOL/no periodontitis rats ($p < 0.05$) (Figure 5R). There was no significant difference in CD68⁺ cell number between ZOL/ONJ and anti-VEGF/periodontitis rats (Figure 5R).

CD31⁺ blood vessels: CD31 is expressed at endothelial cell junctions and used as a marker for blood vessels. CD31⁺ cells associated with vascular structures were found in the lamina propria, periodontal ligament, fibrous tissue, and alveolar bone in maxillary interdental areas between the second and third molars in rats of all groups (Figure 5S-W). In rats with periodontitis, blood vessels were often found in the periodontal ligament and fibrous tissue adjacent to the alveolar bone (Figure 5T, 5V). When CD31⁺ blood vessels were assessed in the complete ROI, there were no significant differences among treatment groups for either maxillae or mandibles (data not shown). However, when the assessment in maxillae was restricted to the interdental area between the second and third molars, there were significantly fewer CD31⁺ blood vessels in anti-VEGFA/periodontitis rats compared to VEH/periodontitis ($p < 0.05$) and ZOL/no periodontitis ($p < 0.05$) rat groups, respectively (Figure 5X). Similarly, when the assessment in mandibles was restricted to the interdental area between the first and second molars, there were significantly fewer CD31⁺ blood vessels in anti-VEGFA rats compared to VEH ($p < 0.05$) and ZOL ($p < 0.05$) rat groups, respectively (Figure 5Y).

Discussion

This is the first preclinical study to investigate whether a medication that inhibits angiogenesis contributes directly to ONJ in rats with periodontitis as a local oral risk factor. In this experiment, rice rats with localized periodontitis given anti-VEGFA antibody monotherapy did not develop ONJ, but rather an extensive inflammatory response in the maxillae and mandibles that resulted in a destructive, advanced form of periodontitis. This form is characterized by extreme alveolar bone loss with fibrosis, pseudoepitheliomatous gingival hyperplasia, and an increased number of CD3⁺ T-cells in the periodontitis-affected tissues. Though necrotic exposed alveolar bone, the hallmark of human ONJ, occurred in concurrently treated ZOL rats, it never occurred in anti-VEGFA rats. These results parallel previous pre-clinical findings with another local oral risk factor for ONJ, tooth extraction, in which an anti-VEGFA antibody delayed healing without causing ONJ [46].

While the data do not explain the occurrence of ONJ in oncology patients taking AgIs, they provide new evidence that may help explain the role of AgIs in ONJ pathogenesis.

Systemically administered AgIs are but one part of oncology treatment regimens. AgIs are frequently combined with cytotoxic chemotherapy (e.g., platinum or taxane-containing medications) [71]. Many oncology patients taking AgIs who develop ONJ have previously been or are concurrently taking powerful anti-resorptives as part of their chemotherapy [14]. In the clinical setting, where systemically administered AgIs are almost always combined with other medications, it is extremely difficult to understand the role of AgIs in ONJ pathogenesis. Monotherapy with a systemically-administered AgI in the rice rat with localized periodontitis provides two potential clarifications to the current understanding of AgIs and jaw abnormalities. First, even though systemically-administered anti-VEGFA monotherapy in rice rats with localized periodontitis does not induce ONJ, it induces a destructive advanced form of periodontitis that may cause functional consequences to the jaw that are as serious as ONJ. Second, this study did not support our working hypothesis that systemically administered AgIs cause ONJ. On the contrary, the data strongly suggest that other agents commonly used with AgIs in cancer chemotherapy protocols, aside from powerful anti-resorptives, should be tested both in monotherapy and combination therapy, for possible roles in ONJ pathogenesis.

The absence of ONJ in anti-VEGFA rats with localized periodontitis indirectly supports an existing powerful anti-resorptive-induced ONJ hypothesis [50, 51] that revolves around the idea that systemic (e.g., medications) and local factors (e.g., tooth extraction, periodontal infection, periapical infection, etc.) must combine to produce ONJ. In the scenario involving the local factor, periodontitis, oral bacteria in concert with an exacerbated host inflammatory response induces irreversible injury and death of alveolar bone. In the absence of systemic factors, the damaged/necrotic alveolar bone is removed so quickly by osteoclastic bone resorption that dead bone is rarely found in such lesions. In the presence of powerful anti-resorptive medications, this hypothesis holds that the osteoclastic resorption of irreversibly-damaged/necrotic alveolar bone tissue that would normally take place, does not occur due to the inhibitory action of powerful anti-resorptives on bone resorption, thus causing necrotic bone tissue to persist in such quantity that it ultimately becomes exposed and accumulates as ONJ [50, 51]. We now hypothesize that anti-VEGFA monotherapy induces a more extreme form of periodontitis than that found in ONJ induced by powerful anti-resorptives. Despite the fact that this destructive advanced periodontitis kills bone, a build-up of necrotic exposed alveolar bone never occurs because normal bone resorption activity is sufficient to remove the irreversibly damaged/necrotic alveolar bone before it can accumulate. In humans, necrotic bone associated with AgIs accumulates only when agents such as powerful anti-resorptives, well-recognized for their strong anti-resorptive activity, or perhaps cytotoxic chemotherapeutic agents which may also have anti-resorptive activity [72, 73] and/or an intrinsic capacity to induce osteonecrosis *per se* are given [74-77].

Our data concerning anti-VEGFA monotherapy may be generally relevant to humans. Worsening of periodontal condition in humans has been reported with combination therapies that include systemic bevacizumab. Periodontitis began after 18 wks. of carboplatin and gemcitabine plus adjuvant bevacizumab, worsened over 10 months, and then stabilized upon bevacizumab cessation [39]. Oncology patients taking cytotoxic chemotherapy with adjuvant systemic bevacizumab display increased severity of periodontitis [40]. While both studies link bevacizumab-based *combination* therapy in humans to worsening of pre-existing

periodontitis, they do not indicate whether bevacizumab *monotherapy* either worsens existing periodontitis or initiates new cases of periodontitis. Findings from our experiment parallel the above clinical studies, except that our experiment omitted cytotoxic chemotherapy and powerful anti-resorptives, suggesting that anti-VEGFA *per se* can worsen pre-existing localized periodontitis. Experiments in rice rats or other pre-clinical models of periodontitis/tooth extraction which test one or more agents applied in chemotherapies (e.g., cytotoxic chemotherapy or powerful anti-resorptive agents) that use an adjuvant AgI, may further improve the understanding of the tissue-level origins of AgI-related periodontitis and ONJ in humans.

The possibility that systemic AgIs might initiate periodontitis in previously periodontally-healthy individuals deserves further comment. In our experiment, anti-VEGFA induced periodontitis in oral cavity regions not affected by localized periodontitis [29, 51]. Anti-VEGFA rats had significantly more mandibular periodontitis than the other two groups. As reported previously [29, 51], VEH and ZOL rats had a low prevalence/severity of mandibular gross lesions that were mostly gingivitis. In contrast, anti-VEGFA rats showed more prevalent and severe mandibular periodontitis, with pseudoepitheliomatous gingival hyperplasia, marked apical migration of the junctional epithelium, marked inflammatory cell infiltration of the lamina propria, disruption of the periodontal ligament, and more alveolar bone loss at both mandibular molar interdental spaces, compared to both VEH and ZOL rats. These findings suggest the possibility that anti-VEGFA monotherapy may initiate new cases of periodontitis. Whether anti-VEGFA directly causes tissue specific alterations or affects the host immune response for maintaining local oral homeostasis is currently unknown. If some forms of cancer chemotherapy can initiate periodontitis in a healthy oral cavity, this would alter the current ONJ paradigm that relies on controlling *pre-existing* local factors to reduce the risk of ONJ [14]. Appropriately-designed pre-clinical studies in gingivitis/periodontitis-free rice rats, other pre-clinical models of periodontitis, or even Sprague-Dawley rats with their natural periodontitis resistance, could shed new light on this problem.

In the host inflammatory response, macrophages differentiate into two functional phenotypes, M1 or M2, depending on the local environmental signals originating from damaged cells, activated lymphocytes, and microorganisms [78, 79]. The M1 phenotype is characterized by production of high levels of pro-inflammatory cytokines, the ability to mediate resistance to pathogens, high production of reactive oxygen species, and promotion of Th1 responses. CD68 is a well-characterized marker of M1 macrophages. In contrast, M2 macrophages are anti-inflammatory, promoting wound healing, tissue remodeling, and immune regulation. We found more CD68+ cells in the periodontium of ZOL rats with ONJ lesions compared to ZOL rats with no periodontitis and VEH rats with or without periodontitis. These data suggest that ZOL, but not anti-VEGFA, enhances the expression of M1 macrophages in the presence of ONJ. These findings are also consistent with recent studies demonstrating that ZOL exacerbates pre-existing inflammation through M1 macrophage polarization [80, 81], and that increased IL-17 is associated with an increased M1:M2 macrophage ratio in mice and humans with ONJ [82]. Studies of pathways activated in early localized periodontal lesions in ZOL and anti-VEGFA rice rats before the onset of periodontitis or ONJ could prove valuable.

If one simply considers the hair/food impaction lesion in the interdental space between the maxillary second and third molars a wound, then our data appear consistent with inhibition of wound healing caused by bevacizumab [46, 83, 84]. These lesions were generally established within 4-6 weeks after the start of study. While they did not progress in VEH rats, they worsened in anti-VEGFA rats. It is known that VEGF treatment significantly enhances macrophage migration and induces M1 macrophages to shift to an M2 phenotype. Hence, it can be inferred that inhibition of VEGF signaling causes a shift in macrophage polarity to the M1 phenotype [85]. This could explain the significantly increased wound healing complications observed in patients that receive bevacizumab adjuvant therapy [86]. Thus, it is reasonable to suggest that anti-VEGFA could have stimulated a shift from M2 toward M1 macrophage polarization, maintaining an intensively active inflammatory phase and inhibiting healing. It seems reasonable to hypothesize that anti-VEGFA monotherapy exacerbates localized periodontitis at the interdental space between the second and third maxillary molars by interfering with wound healing mechanisms directed by VEGF signaling. The different histologic features of the gingival epithelium between ZOL and anti-VEGFA rats cannot be fully explained by the limited analyses conducted here and deserve further investigation. Immunohistochemical analysis also found more CD3+ cells in periodontal tissues of anti-VEGFA rats with destructive advanced periodontitis compared to VEH rats with or without periodontitis and ZOL rats with no periodontitis. The higher prevalence of T-cells in anti-VEGFA rats with periodontitis is consistent with the larger area of tissue involvement that characterized their destructive advanced periodontitis. There were also more CD3+ cells in lesions of anti-VEGFA rats specifically at the interdental space between the second and third maxillary molars, where lesions occur in all three groups. This finding suggests that anti-VEGFA treatment differentially alters the local immune response of oral tissues with pre-existing periodontitis. Establishing the CD4/CD8 T-cell subpopulation ratio would provide further evidence to help explain the histopathological findings observed in anti-VEGFA rats.

Serial oral examinations provided valuable observational data that document the chronology of untreated gingival lesions and open wounds in the maxilla. Incipient gingival lesions became detectable at the lingual aspect of the interdental space between the maxillary second and third molars by age ~9 wks. and persisted unchanged until age 28 weeks in VEH rats. In ZOL rats, the majority (7/9) of such lesions evolved into ONJ near the interdental space between the maxillary second and third molars by age 28 weeks. In anti-VEGFA rats, all the localized periodontal lesions progressed into destructive advanced periodontitis by age 18-22 weeks. These data indicate that both ZOL and anti-VEGFA cause incipient lesions to worsen. With ZOL, the lesions evolved toward ONJ, whereas with anti-VEGF, they evolved into destructive advanced periodontitis. These findings may not only provide a potential warning about how simple oral lesions can deteriorate during treatment with ZOL or anti-VEGFA, but also information that could be used to design studies that investigate temporal aspects of the molecular pathways, pathophysiology, and treatment of such conditions.

Anti-VEGFA rats had a significantly higher serum concentration of VEGFA compared to the non-detectable levels in VEH and ZOL rats. Our data compare well to what is observed in cancer patients treated with anti-VEGF therapy [87-89]. Intravenous administration of

bevacizumab to cancer patients leads to significantly higher serum VEGF [88], which is considered a feedback mechanism and a potential pharmacodynamic marker of VEGF inactivation [87]. Most VEGF in the serum is antibody-bound and hence inactive [90, 91]. The response is generally rapid and reaches near maximal levels within 1-3 days after drug administration [87, 92]. It has been suggested that the serum VEGF rise in cancer patients treated with bevacizumab originates primarily from the accumulation of host-derived VEGF in serum and is explained by antibody interference with receptor-mediated endocytosis and protein degradation [89]. Higher serum VEGF has been reported not only for bevacizumab, but also for other VEGF-targeted approaches such as anti-VEGFR-2 antibodies [87], soluble VEGF receptor competitors [93], and RTKIs [94]. The elevated serum VEGF observed here in anti-VEGFA rats can thus be considered a biomarker of anti-VEGFA activity in this experiment.

Medications implicated in ONJ continue to emerge. ONJ with other AgIs, such as RTK and mTOR inhibitors, has been reported [15]. ONJ in patients exposed to neither powerful anti-resorptives nor AgIs has also been reported [95]. This model of localized periodontitis provided indirect evidence that non-AgI agents used in oncology treatment protocols bear some responsibility for causing ONJ in humans. Future preclinical studies that examine the role of other medications and/or drug combinations associated with ONJ might consider using this rice rat model to improve their understanding and provide a basis for translational studies.

While this study demonstrated novel effects of anti-VEGFA monotherapy on rice rats with localized periodontitis, its limitations should be noted. First, anti-VEGFA treatments began in rice rats aged 4 wks, when bones were growing rapidly. This is dissimilar to the clinical setting, in which AgIs are given mainly to adults. Blocking VEGF in growing mice inhibits angiogenesis in the calcifying cartilage of the growth plate, resulting in thickened growth plates and shortened femurs [96]. However, in the current study, femoral length in anti-VEGFA rats was similar to VEH rats, suggesting that bone elongation processes were not affected by anti-VEGFA. Future studies could consider starting anti-VEGFA when rats have reached full skeletal maturity to avoid potential effects on bone modeling. Second, anti-VEGFA rats had lower body weight compared to VEH rats at wks 18-24 and ZOL rats at wks 20-24. Similar body weight loss was observed in previous rice rat bisphosphonate induced-ONJ studies that was associated with severe oral lesions and reduced food intake [50, 51]. Since anti-VEGFA rats had a high prevalence of severe oral lesions by Week 18 of study, their lower body weight may reflect reduced food intake due to painful mastication, rather than a direct toxic effect of anti-VEGFA. Third, the most accurate vehicle control for B20-4.1.1, IgG antibody, was not used here and should be considered in future experiments. Fourth, anti-VEGFA was administered subcutaneously. This differs from preclinical settings, in which bevacizumab was administered intravenously or intraperitoneally [97]. Previous animal studies have shown that subcutaneous injection may produce species-specific alterations in the pharmacokinetics and pharmacodynamics of B20.4.1.1 [98]. The pharmacokinetic and pharmacodynamics variability specific to rice rats that may result from different administration routes is not known. However, the anti-VEGFA tissue outcomes differed significantly from those of VEH rats, indicating that the therapy affected their oral status. Importantly, in this experiment, serum VEGFA responded in a predictable fashion to

the administration of anti-VEGFA. Additional studies in rice rats to compare oral findings after subcutaneous, intravenous, and intraperitoneal injection would be required to empirically determine if administration route alters the outcome in the oral cavity. Fifth, we used only one dose level of anti-VEGFA that was selected to reflect doses used in oncology patients [87-89, 92]. Although we found no evidence of ONJ, the possibility that ONJ could occur with a higher dose cannot be excluded. Though finding ONJ at higher doses might appear to have utility for building a pre-clinical model, the clinical relevance of such a finding could be questioned, because a dose higher than 5mg/kg 2x/wk is likely to produce a higher serum level of anti-VEGFA activity than occurs in BVZ-treated cancer patients. The possibility that ONJ could occur at 24wks with a lower dose cannot be excluded. With a lower dose, one might envision a lower dose initiating a less intense process that includes alveolar bone death and resorption that would leave periodontitis rats with alveolar bone that is present but exposed and dead, rather than absent, after 24 wks. The less intense disease process could enable studies of its various phases at lower serum levels of anti-VEGFA activity than occur in BVZ-treated cancer patients. Sixth, this study presents necropsy findings about ONJ from only 24wks treatment, a time when pAR-related ONJ is prominent in N-BP-treated rice rats. The possibility that ONJ would have been seen in AgI-treated rats necropsied at earlier times cannot be excluded. Perhaps the process underlying AgI-related ONJ differs from that causing pAR-related ONJ in a way that causes it to develop and evolve more quickly than pAR-related ONJ and then actually resolve by 24 wks into destructive, advanced periodontitis. It is even possible that alveolar bone died and was present long enough to be detected as ONJ at earlier times, but had been completely removed by 24wks, leaving destructive, advanced periodontitis. Though studies of B20.4.1.1 effects in rice rats with periodontitis for shorter necropsy periods could be fruitful, given that many rats already show extensive alveolar bone loss at 24wks, it seems unlikely that longer studies are needed. Seventh, this study did not examine combined ZOL + anti-VEGFA treatment. Having such a group could have allowed better defining the combined roles of anti-VEGFA monotherapy and ZOL, something that occurs in the clinic, in causing ONJ. Pre-clinical experiments in rice rats using AgIs with one or more agents in combination chemotherapies (e.g., cytotoxic or powerful anti-resorptive agents) would be extremely useful.

In conclusion, although ONJ is associated with both AgIs and powerful anti-resorptives in humans, in this animal model, anti-VEGFA monotherapy did not induce ONJ but rather caused a destructive advanced form of periodontitis. This suggests that the medications that are co-administered with anti-VEGFA in oncology patients may be required to cause what is now called AgI-related ONJ. These data have other important implications for understanding the etiology and pathophysiology of periodontitis and ONJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIH grant R01DE023783-01A from the National Institute of Dental and Craniofacial Research (NIDCR). We are thankful to Genentech (San Francisco, CA, USA) for providing the

monoclonal antibody (B20-4.1.1), and to Dr. Juerg Gasser and Novartis Pharma AG (Basel, Switzerland) to provide us with zoledronic acid.

Funding

This research was supported by the National Institute of Dental and Craniofacial Research (NIDCR); R01DE023783-01A.

Abbreviations

AgI	angiogenesis inhibitor
B.Pm	bone perimeter
CEJ	cemento-enamel junction
BV/TV	bone volume
ONJ	Osteonecrosis of the Jaw
MRONJ	medication-related osteonecrosis of the jaw
RANKL	receptor activator of nuclear factor κ B ligand
ROI	region of interest
RTKI	receptor tyrosine kinase inhibitor
VEGF	vascular endothelial growth factor
ZOL	zoledronic acid
STD	standard
GQG	gross quadrant grade
VEH	vehicle
FT	fibrous tissue
BV	blood vessel

References

- [1]. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, Gagel RF, Gilsanz V, Guise T, Koka S, McCauley LK, McGowan J, McKee MD, Mohla S, Pendrys DG, Raisz LG, Ruggiero SL, Shafer DM, Shum L, Silverman SL, Van Poznak CH, Watts N, Woo SB, Shane E, Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research, J Bone Miner. Res 22(10) (2007)1479–1491. [PubMed: 17663640]
- [2]. Bilezikian JP, Osteonecrosis of the jaw--do bisphosphonates pose a risk?, N. Engl. J Med 355(22) (2006) 2278–2281. [PubMed: 17135582]
- [3]. Marx RE, Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic, J Oral Maxillofac. Surg 61(9) (2003) 1115–1117. [PubMed: 12966493]
- [4]. Khan AA, Morrison A, Hanley DA, Felsenberg D, McCauley LK, O'Ryan F, Reid IR, Ruggiero SL, Taguchi A, Tetradis S, Watts NB, Brandi ML, Peters E, Guise T, Eastell R, Cheung AM, Morin SN, Masri B, Cooper C, Morgan SL, Obermayer-Pietsch B, Langdahl BL, Al Dabagh R,

- Davison KS, Kendler DL, Sandor GK, Josse RG, Bhandari M, El Rabbany M, Pierroz DD, Sulimani R, Saunders DP, Brown JP, Compston J, International J Task Force on Osteonecrosis of the, Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus, *J Bone Miner Res* 30(1) (2015) 3–23. [PubMed: 25414052]
- [5]. Stopeck AT, Lipton A, Body JJ, Steger GG, Tonkin K, de Boer RH, Lichinitser M, Fujiwara Y, Yardley DA, Viniegra M, Fan M, Jiang Q, Dansey R, Jun S, Braun A, Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study, *J. Clin. Oncol* 28(35) (2010) 5132–5139. [PubMed: 21060033]
- [6]. Van den Wyngaert T, Wouters K, Huizing MT, Vermorken JB, RANK ligand inhibition in bone metastatic cancer and risk of osteonecrosis of the jaw (ONJ): non bis in idem?, *Support. Care Cancer* 19(12) (2011) 2035–2040. [PubMed: 21203781]
- [7]. Greuter S, Schmid F, Ruhstaller T, Thuerlimann B, Bevacizumab-associated osteonecrosis of the jaw, *Ann Oncol* 19(12) (2008) 2091–2. [PubMed: 18977851]
- [8]. Estilo CL, Fornier M, Farooki A, Carlson D, Bohle G 3rd, Huryn JM, Osteonecrosis of the jaw related to bevacizumab, *J Clin Oncol* 26(24) (2008) 4037–8. [PubMed: 18711196]
- [9]. Disel U, Besen AA, Ozyilkan O, Er E, Canpolat T, A case report of bevacizumab-related osteonecrosis of the jaw: old problem, new culprit, *Oral Oncol* 48(2) (2012) e2–3. [PubMed: 21865077]
- [10]. Katsenos S, Christophylakis C, Psathakis K, Osteonecrosis of the jaw in a patient with advanced non-small-cell lung cancer receiving bevacizumab, *Arch Bronconeumol* 48(6) (2012) 218–9. [PubMed: 22429334]
- [11]. Serra E, Paolantonio M, Spoto G, Mastrangelo F, Tete S, Dolci M, Bevacizumab-related osteonecrosis of the jaw, *Int J Immunopathol Pharmacol* 22(4) (2009) 1121–3. [PubMed: 20074477]
- [12]. Aragon-Ching JB, Ning YM, Chen CC, Latham L, Guadagnini JP, Gulley JL, Arlen PM, Wright JJ, Parnes H, Figg WD, Dahut WL, Higher incidence of Osteonecrosis of the Jaw (ONJ) in patients with metastatic castration resistant prostate cancer treated with anti-angiogenic agents, *Cancer Invest* 27(2) (2009) 221–6. [PubMed: 19235596]
- [13]. Guarneri V, Miles D, Robert N, Dieras V, Glaspy J, Smith I, Thomssen C, Biganzoli L, Taran T, Conte P, Bevacizumab and osteonecrosis of the jaw: incidence and association with bisphosphonate therapy in three large prospective trials in advanced breast cancer, *Breast Cancer Res. Treat* 122(1) (2010) 181–188. [PubMed: 20361252]
- [14]. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, O’Ryan F, American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw--2014 update, *J. Oral Maxillofac. Surg* 72(10) (2014) 1938–1956. [PubMed: 25234529]
- [15]. Nicolatou-Galitis O, Kouri M, Papadopoulou E, Vardas E, Galiti D, Epstein JB, Elad S, Campisi G, Tsoukalas N, Bektas-Kayhan K, Tan W, Body JJ, Migliorati C, Lalla RV, Group MBS, Osteonecrosis of the jaw related to non-antiresorptive medications: a systematic review, *Support Care Cancer* 27(2) (2019) 383–394. [PubMed: 30353228]
- [16]. Rugani P, Walter C, Kirnbauer B, Acham S, Begus-Nahrman Y, Jakse N, Prevalence of Medication-Related Osteonecrosis of the Jaw in Patients with Breast Cancer, Prostate Cancer, and Multiple Myeloma, *Dent J (Basel)* 4(4) (2016).
- [17]. Fliefel R, Troltzsch M, Kuhnisch J, Ehrenfeld M, Otto S, Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review, *Int J Oral Maxillofac Surg* 44(5) (2015) 568–85. [PubMed: 25726090]
- [18]. Lescaille G, Coudert AE, Baaroun V, Ostertag A, Charpentier E, Javelot MJ, Toledo R, Goudot P, Azerad J, Berdal A, Spano JP, Ruhin B, Descroix V, Clinical study evaluating the effect of bevacizumab on the severity of zoledronic acid-related osteonecrosis of the jaw in cancer patients, *Bone* 58 (2014) 103–7. [PubMed: 24120382]
- [19]. Ngamphaiboon N, Frustino JL, Kossoff EB, Sullivan MA, O’Connor TL, Osteonecrosis of the jaw: dental outcomes in metastatic breast cancer patients treated with bisphosphonates with/without bevacizumab, *Clin Breast Cancer* 11(4) (2011) 252–7. [PubMed: 21729657]

- [20]. Christodoulou C, Pervena A, Klouvas G, Galani E, Falagas ME, Tsakalos G, Visvikis A, Nikolakopoulou A, Acholos V, Karapanagiotidis G, Batziou E, Skarlos DV, Combination of bisphosphonates and antiangiogenic factors induces osteonecrosis of the jaw more frequently than bisphosphonates alone, *Oncology* 76(3) (2009) 209–211. [PubMed: 19212145]
- [21]. Badros A, Weikel D, Salama A, Goloubeva O, Schneider A, Rapoport A, Fenton R, Gahres N, Sausville E, Ord R, Meiller T, Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors, *J. Clin. Oncol* 24(6) (2006) 945–952. [PubMed: 16484704]
- [22]. Eleutherakis-Papaiakovou E, Bamias A, Antiresorptive treatment-associated ONJ, *Euro. J. Cancer Care* 20 (2017) 8–24.
- [23]. Carlson ER, Schlott BJ, Anti-resorptive osteonecrosis of the jaws: facts forgotten, questions answered, lessons learned, *Oral. Maxillofac. Surg. Clin. North Am* 26(2) (2014) 171–191. [PubMed: 24630868]
- [24]. Marx RE, Sawatari Y, Fortin M, Broumand V, Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment, *J Oral Maxillofac. Surg* 63(11) (2005) 1567–1575. [PubMed: 16243172]
- [25]. Aghaloo TL, Kang B, Sung EC, Shoff M, Ronconi M, Gotcher JE, Bezouglaia O, Dry SM, Tetradis S, Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat, *J. Bone Miner. Res* 26(8) (2011) 1871–1882. [PubMed: 21351151]
- [26]. Aguirre JJ, Akhter MP, Kimmel DB, Pingel JE, Williams A, Jorgensen M, Kesavalu L, Wronski TJ, Oncologic doses of zoledronic acid induce osteonecrosis of the jaw-like lesions in rice rats (*Oryzomys palustris*) with periodontitis, *J. Bone Miner. Res* 27(10) (2012) 2130–2143. [PubMed: 22623376]
- [27]. Li CL, Lu WW, Seneviratne CJ, Leung WK, Zwahlen RA, Zheng LW, Role of periodontal disease in bisphosphonate-related osteonecrosis of the jaws in ovariectomized rats, *Clin. Oral Implants. Res* 27(1) (2016) 1–6. [PubMed: 25371026]
- [28]. Song M, Alshaikh A, Kim T, Kim S, Dang M, Mehrzarin S, Shin KH, Kang M, Park NH, Kim RH, Preexisting Periapical Inflammatory Condition Exacerbates Tooth Extraction-induced Bisphosphonate-related Osteonecrosis of the Jaw Lesions in Mice, *J. Endod* 42(11) (2016) 1641–1646. [PubMed: 27637460]
- [29]. Messer JG, Jiron JM, Chen HY, Castillo EJ, Mendieta Calle JL, Reinhard MK, Kimmel DB, Aguirre JJ, Prevalence of Food Impaction-Induced Periodontitis in Conventionally Housed Marsh Rice Rats (*Oryzomys palustris*), *Comp Med* 67(1) (2017) 43–50. [PubMed: 28222838]
- [30]. Soutome S, Hayashida S, Funahara M, Sakamoto Y, Kojima Y, Yanamoto S, Umeda M, Factors affecting development of medication-related osteonecrosis of the jaw in cancer patients receiving high-dose bisphosphonate or denosumab therapy: Is tooth extraction a risk factor?, *PLoS One* 13(7) (2018) e0201343. [PubMed: 30048523]
- [31]. O'Halloran M, Boyd NM, Smith A, Denosumab and osteonecrosis of the jaws - the pharmacology, pathogenesis and a report of two cases, *Aust Dent J* 59(4) (2014) 516–9. [PubMed: 25131835]
- [32]. Aljohani S, Gaudin R, Weiser J, Troltsch M, Ehrenfeld M, Kaeppler G, Smeets R, Otto S, Osteonecrosis of the jaw in patients treated with denosumab: A multicenter case series, *J Craniomaxillofac Surg* 46(9) (2018) 1515–1525. [PubMed: 29983309]
- [33]. Kajizono M, Sada H, Sugiura Y, Soga Y, Kitamura Y, Matsuoka J, Sendo T, Incidence and Risk Factors of Osteonecrosis of the Jaw in Advanced Cancer Patients after Treatment with Zoledronic Acid or Denosumab: A Retrospective Cohort Study, *Biol Pharm Bull* 38(12) (2015) 1850–5. [PubMed: 26632176]
- [34]. de Oliveira CC, Brizeno LA, de Sousa FB, Mota MR, Alves AP, Osteonecrosis of the jaw induced by receptor activator of nuclear factor-kappa B ligand (Denosumab) - Review, *Med Oral Patol Oral Cir Bucal* 21(4) (2016) e431–9. [PubMed: 26827069]
- [35]. Abel Mahedi Mohamed H, Nielsen CEN, Schiødt M, Medication related osteonecrosis of the jaws associated with targeted therapy as monotherapy and in combination with antiresorptives. A report of 7 cases from the Copenhagen Cohort, *Oral Surg Oral Med Oral Pathol Oral Radiol* 125(2) (2018) 157–163. [PubMed: 29221983]

- [36]. Saad F, Brown JE, Van Poznak C, Ibrahim T, Stemmer SM, Stopeck AT, Diel IJ, Takahashi S, Shore N, Henry DH, Barrios CH, Facon T, Senecal F, Fizazi K, Zhou L, Daniels A, Carriere P, Dansey R, Incidence, risk factors, and outcomes of osteonecrosis of the jaw: integrated analysis from three blinded active-controlled phase III trials in cancer patients with bone metastases, *Ann. Oncol* 23(5) (2012) 1341–1347. [PubMed: 21986094]
- [37]. de Molon RS, Cheong S, Bezouglaia O, Dry SM, Pirih F, Cirelli JA, Aghaloo TL, Tetradis S, Spontaneous osteonecrosis of the jaws in the maxilla of mice on antiresorptive treatment: a novel ONJ mouse model, *Bone* 68 (2014) 11–19. [PubMed: 25093262]
- [38]. Aghaloo TL, Cheong S, Bezouglaia O, Kostenuik P, Atti E, Dry SM, Pirih FQ, Tetradis S, RANKL inhibitors induce osteonecrosis of the jaw in mice with periapical disease, *J. Bone Miner. Res* 29(4) (2014) 843–854. [PubMed: 24115073]
- [39]. Gujral DM, Bhattacharyya S, Hargreaves P, Middleton GW, Periodontal disease in a patient receiving Bevacizumab: a case report, *J Med Case Rep* 2 (2008) 47. [PubMed: 18271967]
- [40]. Ogawa K, Ueno T, Kato K, Nishitani H, Akiyoshi K, Iwasa S, Nakajima TE, Hamaguchi T, Yamada Y, Hosokawa A, Sugiyama T, Shimada Y, A retrospective analysis of periodontitis during bevacizumab treatment in metastatic colorectal cancer patients, *Int J Clin Oncol* 18(6) (2013) 1020–4. [PubMed: 23011103]
- [41]. Magremanne M, Lahon M, De Ceulaer J, Reyckler H, Unusual bevacizumab-related complication of an oral infection, *J Oral Maxillofac Surg* 71(1) (2013) 53–5. [PubMed: 22705223]
- [42]. Gilabert M, Provansal M, Cappiello M, Walz Y, Salem N, Tarpin C, Brunelle S, Thomassin J, Gravis G, Buccodental side effects of sunitinib in patients with metastatic renal cell carcinoma, *Br J Cancer* 109(7) (2013) 1750–4. [PubMed: 24045668]
- [43]. Hoefert S, Eufinger H, Sunitinib may raise the risk of bisphosphonate-related osteonecrosis of the jaw: presentation of three cases, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 110(4) (2010) 463–9. [PubMed: 20692189]
- [44]. Pereira-Lopes O, Sampaio-Maia B, Sampaio S, Vieira-Marques P, Monteiro-da-Silva F, Braga AC, Felino A, Pestana M, Periodontal inflammation in renal transplant recipients receiving everolimus or tacrolimus - preliminary results, *Oral Dis* 19(7) (2013) 666–72. [PubMed: 23448098]
- [45]. Pakosch D, Papadimas D, Munding J, Kawa D, Kriwalsky MS, Osteonecrosis of the mandible due to anti-angiogenic agent, bevacizumab, *Oral Maxillofac Surg* 17(4) (2013) 303–6. [PubMed: 23242941]
- [46]. Akita Y, Kuroshima S, Nakajima K, Hayano H, Kanai R, Sasaki M, Sawase T, Effect of anti-angiogenesis induced by chemotherapeutic monotherapy, chemotherapeutic/bisphosphonate combination therapy and anti-VEGFA mAb therapy on tooth extraction socket healing in mice, *J Bone Miner Metab* 36(5) (2018) 547–559. [PubMed: 29043461]
- [47]. Gotcher JE, Jee WS, The progress of the periodontal syndrome in the rice rat. I. Morphometric and autoradiographic studies, *J Periodontal Res* 16(3) (1981) 275–291. [PubMed: 6458677]
- [48]. Gupta O, Shaw J, Periodontal disease in the rice rat. I. Anatomic and histopathologic findings., *Oral Surg. Oral Med Oral Pathol* 9(6) (1956) 592–603. [PubMed: 13322421]
- [49]. Aguirre JI, Akhter M, Kimmel D, Pingel J, Xia X, Williams A, Jorgensen M, Edmonds K, Lee J, Reinhard M, Battles A, Kesavalu L, Wronski TJ, Enhanced alveolar bone loss in a model of non-invasive periodontitis in rice rats, *Oral Dis* 18(5) (2012) 459–468. [PubMed: 22233442]
- [50]. Messer JG, Mendieta Calle JL, Jiron JM, Castillo EJ, Van Poznak C, Bhattacharyya N, Kimmel DB, Aguirre JI, Zoledronic acid increases the prevalence of medication-related osteonecrosis of the jaw in a dose dependent manner in rice rats (*Oryzomys palustris*) with localized periodontitis, *Bone* 108 (2018) 79–88. [PubMed: 29289789]
- [51]. Messer JG, Jiron JM, Mendieta Calle JL, Castillo EJ, Israel R, Phillips EG, Yarrow JF, Van Poznak C, Kesavalu L, Kimmel DB, Aguirre JI, Zoledronate Treatment Duration Is Linked to Bisphosphonate-Related ONJ Prevalence in Rice Rats with Generalized Periodontitis, *Oral Dis* (2019).

- [52]. Gotcher JE, Jee WS, The progress of the periodontal syndrome in the rice rat. II. The effects of a diphosphonate on the periodontium, *J Periodontal Res* 16(4) (1981) 441–455. [PubMed: 6459441]
- [53]. Du H, Gao M, Qi C, Liu S, Lin Y, Drug-induced gingival hyperplasia and scaffolds: they may be valuable for horizontal food impaction, *Med. Hypotheses* 74(6) (2010) 984–985. [PubMed: 20172659]
- [54]. Matthews DC, Tabesh M, Detection of localized tooth-related factors that predispose to periodontal infections, *Periodontol.* 2000 34 (2004) 136–150. [PubMed: 14717860]
- [55]. Bagri A, Berry L, Gunter B, Singh M, Kasman I, Damico LA, Xiang H, Schmidt M, Fuh G, Hollister B, Rosen O, Plowman GD, Effects of anti-VEGF treatment duration on tumor growth, tumor regrowth, and treatment efficacy, *Clin Cancer Res* 16(15) (2010) 3887–900. [PubMed: 20554752]
- [56]. Singh M, Couto SS, Forrest WF, Lima A, Cheng JH, Molina R, Long JE, Hamilton P, McNutt A, Kasman I, Nannini MA, Reslan HB, Cao TC, Ho CC, Barck KH, Carano RA, Foreman O, Eastham-Anderson J, Jubb AM, Ferrara N, Johnson L, Anti-VEGF antibody therapy does not promote metastasis in genetically engineered mouse tumour models, *J Pathol* 227(4) (2012) 417–30. [PubMed: 22611036]
- [57]. Raina S, Honer M, Kramer SD, Liu Y, Wang X, Segerer S, Wuthrich RP, Serra AL, Anti-VEGF antibody treatment accelerates polycystic kidney disease, *Am J Physiol Renal Physiol* 301(4) (2011) F773–83. [PubMed: 21677148]
- [58]. Edrei Y, Gross E, Corchia N, Abramovitch R, Improved efficacy of a novel anti-angiogenic drug combination (TL-118) against colorectal-cancer liver metastases; MRI monitoring in mice, *Br J Cancer* 107(4) (2012) 658–66. [PubMed: 22805330]
- [59]. Riesner K, Shi Y, Jacobi A, Krater M, Kalupa M, McGearey A, Mertlitz S, Cordes S, Schrenzenmeier JF, Mengwasser J, Westphal S, Perez-Hernandez D, Schmitt C, Dittmar G, Guck J, Penack O, Initiation of acute graft-versus-host disease by angiogenesis, *Blood* 129(14) (2017) 2021–2032. [PubMed: 28096092]
- [60]. Mangani D, Weller M, Seyed Sadr E, Willscher E, Seystahl K, Reifenberger G, Tabatabai G, Binder H, Schneider H, Limited role for transforming growth factor-beta pathway activation-mediated escape from VEGF inhibition in murine glioma models, *Neuro Oncol* 18(12) (2016) 1610–1621. [PubMed: 27286797]
- [61]. Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, Fu L, Malik AK, Gerber HP, Ferrara N, Fuh G, Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF, *J Biol Chem* 281(2) (2006) 951–61. [PubMed: 16278208]
- [62]. Jiron JM, Mendieta Calle JL, Castillo EJ, Abraham AM, Messer JG, Malphurs WL, Malinowski C, Grove K, Reznikov LR, Zubcevic J, Aguirre JI, Comparison of Isoflurane, Ketamine-Dexmedetomidine, and Ketamine-Xylazine for General Anesthesia during Oral Procedures in Rice Rats (*Oryzomys palustris*), *J Am Assoc Lab Anim Sci* 58(1) (2019) 40–49. [PubMed: 30572978]
- [63]. Fondi C, Franchi A, Definition of bone necrosis by the pathologist, *Clin. Cases. Miner. Bone Metab* 4(1) (2007) 21–26. [PubMed: 22460748]
- [64]. Franco-Pretto E, Pacheco M, Moreno A, Messa O, Gnecco J, Bisphosphonate-induced osteonecrosis of the jaws: clinical, imaging, and histopathology findings, *Oral Surg Oral Med Oral Pathol Oral Radiol* 118(4) (2014) 408–17. [PubMed: 25179128]
- [65]. Zheng LZ, Wang JL, Kong L, Huang L, Tian L, Pang QQ, Wang XL, Qin L, Steroid-associated osteonecrosis animal model in rats, *J Orthop Translat* 13 (2018) 13–24. [PubMed: 29662787]
- [66]. Yang L, Boyd K, Kaste SC, Kamdem Kamdem L, Rahija RJ, Relling MV, A mouse model for glucocorticoid-induced osteonecrosis: effect of a steroid holiday, *J Orthop Res* 27(2) (2009) 169–75. [PubMed: 18683891]
- [67]. Kuroshima S, Yamashita J, Chemotherapeutic and antiresorptive combination therapy suppressed lymphangiogenesis and induced osteonecrosis of the jaw-like lesions in mice, *Bone* 56(1) (2013) 101–109. [PubMed: 23727433]

- [68]. Yamashita J, Koi K, Yang DY, McCauley LK, Effect of zoledronate on oral wound healing in rats, Clin. Cancer Res 17(6) (2011) 1405–1414. [PubMed: 21149614]
- [69]. Aguirre JI, Akhter MP, Neuville KG, Trcalek CR, Leeper AM, Williams AA, Rivera M, Kesavalu L, Ke HZ, Liu M, Kimmel DB, Age-related periodontitis and alveolar bone loss in rice rats, Arch. Oral Biol 73 (2016) 193–205. [PubMed: 27771588]
- [70]. Aguirre JI, Leal ME, Rivera MF, Vanegas SM, Jorgensen M, Wronski TJ, Effects of basic fibroblast growth factor and a prostaglandin E2 receptor subtype 4 agonist on osteoblastogenesis and adipogenesis in aged ovariectomized rats, J Bone Miner. Res 22(6) (2007) 877–888. [PubMed: 17352655]
- [71]. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, Mannel RS, Homesley HD, Fowler J, Greer BE, Boente M, Birrer MJ, Liang SX, Gynecologic Oncology G, Incorporation of bevacizumab in the primary treatment of ovarian cancer, N Engl J Med 365(26) (2011) 2473–83. [PubMed: 22204724]
- [72]. Ang ES, Pavlos NJ, Chim SM, Feng HT, Scaife RM, Steer JH, Zheng MH, Xu J, Paclitaxel inhibits osteoclast formation and bone resorption via influencing mitotic cell cycle arrest and RANKL-induced activation of NF-kappaB and ERK, J Cell Biochem 113(3) (2012) 946–55. [PubMed: 22034016]
- [73]. Takahashi M, Mizoguchi T, Uehara S, Nakamichi Y, Yang S, Naramoto H, Yamashita T, Kobayashi Y, Yamaoka M, Furusawa K, Udagawa N, Uematsu T, Takahashi N, Docetaxel inhibits bone resorption through suppression of osteoclast formation and function in different manners, J Bone Miner Metab 27(1) (2009) 24–35. [PubMed: 19082914]
- [74]. Shim K, MacKenzie MJ, Winkquist E, Chemotherapy-associated osteonecrosis in cancer patients with solid tumours: a systematic review, Drug Saf 31(5) (2008) 359–71. [PubMed: 18422377]
- [75]. Infante-Cossio P, Lopez-Martin JC, Gonzalez-Cardero E, Martinez-de-Fuentes R, Casas-Fernandez-Tejerina A, Osteonecrosis of the maxilla associated with cancer chemotherapy in patients wearing dentures, J Oral Maxillofac Surg 70(7) (2012) 1587–92. [PubMed: 22177825]
- [76]. Fan C, Foster BK, Wallace WH, Xian CJ, Pathobiology and prevention of cancer chemotherapy-induced bone growth arrest, bone loss, and osteonecrosis, Curr Mol Med 11(2) (2011) 140–51. [PubMed: 21342129]
- [77]. Hsu HC, Liao TY, Chen DW, Juan YH, Liaw CC, Avascular Necrosis of Bone following Chemotherapy in Cancer Patients with Coagulopathy: Report of Two Cases, Case Rep Oncol 11(1) (2018) 185–190. [PubMed: 29681819]
- [78]. Murray PJ, Macrophage Polarization, Annu Rev Physiol 79 (2017) 541–566. [PubMed: 27813830]
- [79]. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A, Macrophage plasticity, polarization, and function in health and disease, J Cell Physiol 233(9) (2018) 6425–6440.
- [80]. Zhu W, Xu R, Du J, Fu Y, Li S, Zhang P, Liu L, Jiang H, Zoledronic acid promotes TLR-4-mediated M1 macrophage polarization in bisphosphonate-related osteonecrosis of the jaw, FASEB J 33(4) (2019) 5208–5219. [PubMed: 30624969]
- [81]. Kaneko J, Okinaga T, Hikiji H, Ariyoshi W, Yoshiga D, Habu M, Tominaga K, Nishihara T, Zoledronic acid exacerbates inflammation through M1 macrophage polarization, Inflamm Regen 38 (2018) 16. [PubMed: 29977413]
- [82]. Zhang Q, Atsuta I, Liu S, Chen C, Shi S, Shi S, Le AD, IL-17-mediated M1/M2 macrophage alteration contributes to pathogenesis of bisphosphonate-related osteonecrosis of the jaws, Clin Cancer Res 19(12) (2013) 3176–88. [PubMed: 23616636]
- [83]. Gordon CR, Rojavin Y, Patel M, Zins JE, Grana G, Kann B, Simons R, Atabek U, A review on bevacizumab and surgical wound healing: an important warning to all surgeons, Ann Plast Surg 62(6) (2009) 707–9. [PubMed: 19461291]
- [84]. Ahn JW, Shalabi D, Correa-Selm LM, Dasgeb B, Nikbakht N, Cha J, Impaired wound healing secondary to bevacizumab, Int Wound J 16(4) (2019) 1009–1012. [PubMed: 31111622]
- [85]. Wheeler KC, Jena MK, Pradhan BS, Nayak N, Das S, Hsu CD, Wheeler DS, Chen K, Nayak NR, VEGF may contribute to macrophage recruitment and M2 polarization in the decidua, PLoS One 13(1) (2018) e0191040. [PubMed: 29324807]

- [86]. Koskas M, Chereau E, Ballester M, Selle F, Rouzier R, Darai E, Wound complications after bevacizumab treatment in patients operated on for ovarian cancer, *Anticancer Res* 30(11) (2010) 4743–7. [PubMed: 21115934]
- [87]. Bocci G, Man S, Green SK, Francia G, Ebos JM, du Manoir JM, Weinerman A, Emmenegger U, Ma L, Thorpe P, Davidoff A, Huber J, Hicklin DJ, Kerbel RS, Increased plasma vascular endothelial growth factor (VEGF) as a surrogate marker for optimal therapeutic dosing of VEGF receptor-2 monoclonal antibodies, *Cancer Res* 64(18) (2004) 6616–25. [PubMed: 15374976]
- [88]. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA, A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer, *N Engl J Med* 349(5) (2003) 427–34. [PubMed: 12890841]
- [89]. Alidzanovic L, Starlinger P, Schauer D, Maier T, Feldman A, Buchberger E, Stift J, Koeck U, Pop L, Gruenberger B, Gruenberger T, Brostjan C, The VEGF rise in blood of bevacizumab patients is not based on tumor escape but a host-blockade of VEGF clearance, *Oncotarget* 7(35) (2016) 57197–57212. [PubMed: 27527865]
- [90]. Starlinger P, Alidzanovic L, Schauer D, Maier T, Nemeth C, Perisanidis B, Tamandl D, Gruenberger B, Gruenberger T, Brostjan C, Neoadjuvant bevacizumab persistently inactivates VEGF at the time of surgery despite preoperative cessation, *Br J Cancer* 107(6) (2012) 961–6. [PubMed: 22850548]
- [91]. Loupakis F, Falcone A, Masi G, Fioravanti A, Kerbel RS, Del Tacca M, Bocci G, Vascular endothelial growth factor levels in immunodepleted plasma of cancer patients as a possible pharmacodynamic marker for bevacizumab activity, *J Clin Oncol* 25(13) (2007) 1816–8. [PubMed: 17470880]
- [92]. Willett CG, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY, Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients, *J Clin Oncol* 23(31) (2005) 8136–9. [PubMed: 16258121]
- [93]. Schmitz V, Vilanueva H, Raskopf E, Hilbert T, Barajas M, Dzienisowicz C, Gorschluter M, Strehl J, Rabe C, Sauerbruch T, Prieto J, Caselmann WH, Qian C, Increased VEGF levels induced by anti-VEGF treatment are independent of tumor burden in colorectal carcinomas in mice, *Gene Ther* 13(16) (2006) 1198–205. [PubMed: 16617302]
- [94]. Deprimo SE, Bello CL, Smeraglia J, Baum CM, Spinella D, Rini BI, Michaelson MD, Motzer RJ, Circulating protein biomarkers of pharmacodynamic activity of sunitinib in patients with metastatic renal cell carcinoma: modulation of VEGF and VEGF-related proteins, *J Transl Med* 5 (2007) 32. [PubMed: 17605814]
- [95]. Aghaloo TL, Tetradis S, Osteonecrosis of the Jaw in the Absence of Antiresorptive or Antiangiogenic Exposure: A Series of 6 Cases, *J. Oral Maxillofac. Surg* 75(1) (2017) 129–142. [PubMed: 27569557]
- [96]. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N, VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation, *Nat Med* 5(6) (1999) 623–8. [PubMed: 10371499]
- [97]. Selvaraj S, Raundhal M, Patidar A, Saha B, Anti-VEGF antibody enhances the antitumor effect of CD40, *Int J Cancer* 135(8) (2014) 1983–8. [PubMed: 24604357]
- [98]. Lin YS, Nguyen C, Mendoza JL, Escandon E, Fei D, Meng YG, Modi NB, Preclinical pharmacokinetics, interspecies scaling, and tissue distribution of a humanized monoclonal antibody against vascular endothelial growth factor, *J Pharmacol Exp Ther* 288(1) (1999) 371–8. [PubMed: 9862791]

Highlights

- Rice rats with localized maxillary periodontitis that received anti-vascular endothelial growth factor A (anti-VEGF) antibody monotherapy developed destructive advanced periodontitis.
- Rice rats with localized maxillary periodontitis that received zoledronic acid (ZOL) monotherapy developed osteonecrosis of the jaw (ONJ).
- In the mandible, rice rats given anti-VEGF antibody monotherapy developed mild to severe periodontitis.
- Anti-VEGF antibody monotherapy did not cause ONJ in a validated ZOL/ONJ pre-clinical model, but did cause destructive advanced periodontitis.

Figure 1. Prevalence and characteristics of gross oral lesions

A. Prevalence of rats with maxillary lesions during oral exams [*in vivo* gross quadrant grade (GQG)] rose quickly by 3-7 wks in all groups and remained relatively stable thereafter. Prevalence of rats with maxillary lesions tended to be higher in the anti-VEGFA group compared to the VEH group during wks 17-23 ($p = 0.06$). **B.** Maxillary lesion severity during oral exams (*in vivo* GQG) was affected by both duration ($p < 0.001$) and treatment ($p = 0.034$). Though severity in the VEH group remained stable, it worsened with time in both ZOL and anti-VEGFA groups. The anti-VEGFA group had significantly worse lesions compared to the VEH group starting around 13 wks. The ZOL group never differed significantly from either the anti-VEGFA or VEH groups. $*p > 0.05$; Mean $\pm$ SD. **C.** Highest GQG maxillary lesion per rat Ex vivo (24 weeks). Gross lesions were more prevalent in

ZOL and anti-VEGFA rats compared to VEH rats (* $p = 0.017$; * $p = 0.001$, respectively), and gross lesions were more prevalent in anti-VEGFA compared to ZOL rats ($\dagger p = 0.025$). **D.** Photographs of representative maxillae with healthy (GQG=0), mild (GQG=2), moderate (GQG=3), and severe (GQG=4) lesions. GQG 2 lesions appeared to be open wounds. Note gingival/oral mucosal recession around molars in both ZOL and anti-VEGFA groups. Spontaneous tooth loss (note left second molar in GQG4 rat) or migration (right second molar in GQG4 rat) also occurred occasionally in both groups. **E.** Rat Mandibular GQG (24 weeks). There tended to be a higher prevalence of rats with mandibular lesions in the anti-VEGFA group compared to the VEH ($p = 0.08$) and ZOL groups ($p = 0.08$), while ZOL and VEH groups did not differ.

Figure 2. Histopathologic assessment of necrotic exposed bone and other histopathologic features Representative photomicrographs of maxillae of three different rats from the ZOL (A-C) and anti-VEGF (D-F) groups. **a-c** and **d-f** are photomicrographs of areas outlined in black in A-C and D-F, at two-fold higher magnification. Maxillary oral lesions at the palate (A) and interdental space between the second and third molars, in proximity to the lingual (B) and buccal (C) surfaces in a ZOL rat. Exposed, necrotic bone is present in both the hard palate and the interdental space (demarcated in yellow). The gingival epithelium (GE) is ulcerated and the bone is exposed. **a-c**. Alveolar bone with numerous adjacent osteocyte lacunae that contained either no osteocyte nuclei, pyknotic nuclei, or nuclear debris was present (yellow arrow heads). Exposed, necrotic bone was found only in ZOL rats. Oral lesions at the palate (D) and interdental space between the second and third maxillary molars, in proximity to the

lingual (**E**) and buccal (**F**) surfaces in an anti-VEGFA rat. Alveolar bone, when present, was always vital and covered by gingival epithelium. **d-f**. Higher magnification shows vital bone with osteocyte lacunae occupied by basophilic nuclei and overlying fibrous tissue and inflammatory infiltrate. When alveolar bone was present, it never contained fields of adjacent empty osteocyte lacunae. The percentage of empty osteocyte lacunae (**G**), and the number of empty osteocyte lacunae per mm² of bone area (B.Ar) (**H**) were significantly higher in ZOL rats with ONJ compared to VEH (* $p < 0.05$) or anti-VEGFA rats (* $p < 0.05$) with periodontitis. **I**. Maxillary PD Score for each rat. 93% of VEH rats, 100% of ZOL rats, and 93% of anti-VEGFA rats had histopathologic lesions. However, PD Scores in VEH rats ranged only from gingivitis (PD Score 1) to mild-moderate periodontitis (PD Score 2-3; periodontitis). In contrast, 80% of anti-VEGFA rats had destructive, advanced periodontitis (PD Score 4) (**I**.) There was a significantly higher prevalence of anti-VEGFA rats with moderate to severe periodontitis (PD Scores 3) compared to VEH rats (* $p = 0.025$). Though 50% of ZOL rats had ONJ, ONJ was never found in VEH or anti-VEGFA rats. Hence, prevalence of ONJ in ZOL rats was significantly higher than in VEH ($\dagger p = 0.002$) and anti-VEGFA rats ($\dagger p = 0.002$). **J-L**. Representative photomicrographs of lesions showing major histopathologic abnormalities in anti-VEGFA rats. The gingival epithelium rats frequently displayed pseudoepitheliomatous hyperplasia, in which tongue-like projections ($\ddagger$) of the gingival epithelium extend into the lamina propria and PDL at interdental spaces (M) (**J**.) Marked apical migration of the junctional epithelium with nearly complete exposure of the tooth roots in the interdental spaces was also observed. Severe alveolar bone loss with proliferation of fibrous tissue (FT) and PDL that replaced the resorbed bone was present. Marked inflammatory cell infiltration and abundant blood vessels were also present in the lamina propria and PDL (**J-K**). **M-O**. While gingival epithelial hyperplasia (black arrows) was also observed in ZOL rats with exposed necrotic bone, the pseudoepitheliomatous form was less frequent. The alveolar bone of ZOL rats had numerous and prominent cement lines (yellow arrows) (**N-O**). The maxillary alveolar bone that remained in anti-VEGFA rats appeared normal and without the basophilic ground substance seen in ZOL rats (**L**).

Figure 3. Quantification of maxillary and mandibular alveolar bone loss

A-B. Interdental maxillary alveolar bone loss. Maxillary alveolar bone loss was significantly greater at the interdental space between the second and third molars (M2M3) in the VEH/periodontitis, ZOL/ONJ, and anti-VEGF/periodontitis groups compared to the VEH/no periodontitis, ZOL/no periodontitis, and anti-VEGF/no periodontitis groups, respectively ($*p < 0.01$) (**A**). Maxillary alveolar bone loss was also significantly greater in anti-VEGF/periodontitis rats compared to VEH/periodontitis rats ($\dagger p < 0.001$) and ZOL/ONJ rats ($\ddagger p < 0.001$). At the interdental space between the maxillary first and second molars (M1M2), alveolar bone loss was significantly greater in anti-VEGF/periodontitis rats than in all other groups ($\ddagger p < 0.05$) (Mean $\pm$ SD) (**B**). **C-F.** Representative images demonstrating the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest at the M2M3 interdental

space in VEH/no periodontitis (**C**), VEH/periodontitis (**D**), ZOL/ONJ (**E**), and anti-VEGF/periodontitis (**F**) groups. **G**. Mandibular PD Score for each rat. Most VEH and ZOL rats had gingivitis (PD Score 1). In contrast, 47% of anti-VEGFA rats had mild to severe periodontitis (PD Score 2-4) (* $p < 0.05$ compared to VEH and ZOL, respectively). **H**. Alveolar bone loss was significantly greater at the mandibular M1M2 interdental space in the anti-VEGFA group compared to VEH or ZOL groups ($\dagger p < 0.05$, respectively). Alveolar bone loss was significantly greater at the M2M3 interdental space in anti-VEGFA rats compared to VEH rats (* $p < 0.05$). **I-L**. Representative photomicrographs of mandibular interdental spaces. Gingivitis in VEH (**I**) and ZOL rats (**J**), showing plaque on the gingival epithelium and tooth surfaces (*), and migration of the junctional epithelium (black arrows). The alveolar bone and periodontal ligament appear normal. Representative microphotographs of anti-VEGFA rats depicting more severe lesions with marked junctional epithelial migration with root exposure, disruption of normal periodontal ligament architecture, alveolar bone resorption, and abundant plaque at tooth surfaces (**K-L**).

Figure 4. Assessment of bone structure by MicroCT

A-E. Representative three-dimensional reconstructions of the maxillae (lingual view) from each group are shown. Maxillae from the VEH group with no periodontitis (**A**) and with periodontitis (**B**). Note greater alveolar bone loss localized to the interdental space between the maxillary second and third molars (lesion demarcated in yellow) in (**B**). Maxilla from a ZOL rat with ONJ (**C**) showing locally-increased alveolar bone loss at the interdental space between the second and third molars, as well as honeycomb like bone architecture (white arrowheads) extending onto the palatal bone distal to the necrotic area. Maxillae from anti-VEGFA rats without periodontitis (**D**) and with destructive advanced periodontitis (**E**) characterized by severe alveolar bone loss and absence of bony tooth support. Lesion extends into both interdental spaces.

Figure 5. *in situ* analysis of TRAP⁺ osteoclasts, T cells (CD3⁺ cells), cells of the monocyte lineage (CD68⁺ cells) and blood vessels (CD 31⁺) in oral lesions of rats from the different experimental groups by immunohistochemistry.

Representative photomicrographs of TRAP⁺ cells onto alveolar bone surfaces of maxillae from VEH/no periodontitis (A), VEH/periodontitis (B), ZOL/no periodontitis (C), ZOL/ONJ (D), and anti-VEGF/periodontitis (E). There were significantly more TRAP⁺ cells in VEH/periodontitis, ZOL/ONJ, and anti-VEGF/periodontitis compared to VEH/no periodontitis (* $p < 0.05$) and ZOL/no periodontitis († $p < 0.05$). There were no significant differences among lesion-bearing rats (F). CD3⁺ cells were predominantly found in the gingival epithelium (GE) and lamina propria (LP) of VEH/no periodontitis rats (G), VEH/periodontitis (H), and ZOL/no periodontitis (I) rats. ZOL/ONJ rats showed CD3⁺ cells in the gingival epithelium, lamina propria, and fibrous tissue (FT) surrounding necrotic bone.

(demarcated in yellow) (**J**). Anti-VEGF/periodontitis rats had CD3⁺ cells in the gingival epithelium and lamina propria, as well as in the underlying FT (**K**). There were significantly more CD3⁺ cells in the anti-VEGF/periodontitis rats compared to VEH/no periodontitis, VEH/periodontitis, and ZOL/no periodontitis rats (* $p < 0.05$, respectively) (**L**). There tended to be more CD3⁺ cells in anti-VEGFA rats with periodontitis compared to ZOL rats with ONJ (**L**). CD68⁺ cells were found in the lamina propria, PDL, surrounding blood vessels (BV), and at bone surfaces of all treatment groups (**M-R**). There were significantly more CD68⁺ cells in ZOL/ONJ rats compared to VEH/no periodontitis rats, VEH/periodontitis rats, and ZOL/no periodontitis rats (* $p < 0.05$, respectively) (**R**). There were no significant differences in the number of CD68⁺ cells between ZOL/ONJ rats and anti-VEGF/periodontitis rats (**R**). CD31⁺ cells associated with vascular structures were labeled in the lamina propria, PDL, fibrous tissue (FT), and alveolar bone. In rats with lesions, blood vessels were often found in the PDL and FT adjacent to the alveolar bone (**S-W**). There were significantly fewer CD31⁺ blood vessels in anti-VEGFA/periodontitis rats compared to VEH/periodontitis ($p < 0.05$) and ZOL/no periodontitis ($p < 0.05$) in the interdental area between the second and third maxillary molars (**X**). Similarly, when the assessment in mandibles was restricted to the interdental area between the first and second molars, there were significantly fewer CD31⁺ blood vessels in anti-VEGFA rats compared to VEH ($p < 0.05$) and ZOL ($p < 0.05$) rat groups, respectively (**Y**).

Table 1.Criteria for Gross Quadrant Grades (*GQG*)

GQG	Degree	Gross Lesion Description
0	absence	None
0.5	incipient	Hair strands and food particles at an interdental space with no soft tissue inflammation.
1	slight	Discrete lesion in an interdental space; minimal gingival recession; impacted materials (hair, food debris); inflammation at margin of impacted materials.
2	mild	Single open wound lesion in an interdental space with ulceration/recession of lingual gingiva along 1/2 to 2/3 the lingual gingival margin of one involved molar; limited extension into the lingual mucosa; OR presence of two separate <i>GQG</i> =1 lesions within the same quadrant; inflammation/swelling/redness at margin of impacted material.
3	moderate	Open wound involving recession/ulceration of gingiva along entire lingual gingival margin of both involved molars; ulceration extending into lingual mucosa towards midline in maxilla or onto lingual mucosa (lingual plate) in mandible; inflammation/ swelling/redness at margin of impacted material; possible involvement of buccal mucosa.
4	severe	Open wound involving gingival recession/ulceration around all three molars; ulceration extending into lingual mucosa toward the midline and onto the buccal mucosa; marked gingival inflammation/swelling/redness along lingual gingival margin of all molars; possible tooth migration or loss.

Table 2.

Histopathologic PD Score (Inflammation scoring system)

PD Score	Degree	Description
0	absence	None
1	slight	Gingivitis: slight hyperplasia of the gingival epithelium, intraepithelial inflammatory cell infiltration. Modest bacterial plaque accumulation. Normal lamina propria, periodontal ligament and alveolar bone.
2	mild	Periodontitis: gingival hyperplasia and slight migration of junctional epithelium at interdental space. Moderate accumulation of bacterial plaque and hair/food debris. Limited buccolingual extension of lesion. Inflammatory cell infiltration of the gingival epithelium, lamina propria, and periodontal ligament. Mild disruption of periodontal ligament and mild loss of alveolar bone.
3	moderate	Gingival hyperplasia, marked apical migration of junctional epithelium at interdental region. Marked accumulation of bacterial plaque and hair/food debris. Buccolingual extension of lesion. Moderate inflammatory cell infiltration of lamina propria, disruption of periodontal ligament, and moderate loss of alveolar bone.
4	destructive, advanced	Pseudoepitheliomatous gingival hyperplasia, marked apical migration of junctional epithelium with near full root exposure or exposure of bifurcation at interdental region. Marked accumulation of bacterial plaque and hair/food debris. Marked extension of lesion in buccolingual and mesiodistal directions. Marked inflammatory cell infiltration of lamina propria, disruption of periodontal ligament, and frequent near absence of alveolar bone and extensive fibrosis.
ONJ		Exposed necrotic bone, with or without colonization of bacterial colonies directly on bone surfaces. Lack of overlying gingival epithelium and lamina propria. Severe destruction of the periodontium, fibrosis and inflammatory cell infiltration of soft oral tissues.

Table 3.Prevalence of Quadrants with Gross Lesions[/]

Treatment	% Mx Quadrants with Lesions	Fisher Exact <i>p</i> -value	% Mn Quadrants with Lesions	Fisher Exact <i>p</i> -value	% of All Quadrants with Lesions	Chi-Square (<i>p</i> -value)
VEH	27	-	3	-	15	-
ZOL	43	0.270 [*]	4	1.000 [*]	23	0.373 [*]
Ant-VEGFA	73	<0.001 [*] 0.032 [†]	37	0.002 [*] 0.003 [†]	55	<0.001 [*] <0.001 [†]

[/] Data in this table express number of maxillary and mandibular quadrants with lesions (total number of quadrants assessed=180).^{*} vs. VEH[†] vs. ZOL

Mx- maxilla; Mn- mandible