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Journal of Dental Science, Oral and Maxillofacial Research

Bisphosphonate-induced increase in THBS1, TIMP1, and NRP2 mRNA decreases angiogenesis and induces medication-related osteonecrosis of the jaws (MRONJ)

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Received: December 10, 2020 | **Published:** January 26, 2021

Abstract

Medication related osteonecrosis of the jaw (MRONJ) is a disorder characterized by ischemia and death to bones of the jaws. MRONJ is found in a sub-group of patients undergoing bisphosphonate or other drug therapy for osteoporosis or the hypercalcemia associated with certain cancers. In our previous studies, we created a rat model of MRONJ by two injections of 60ug/Kg zoledronic acid (ZA) via tail vein followed by extraction of a single first molar. In this model we have shown that the vasculature of the jaws is diminished relative to controls and that bone healing is delayed.

Objective: The current study was designed to introduce a novel pathway for the diminished angiogenesis found in MRONJ. This pathway involves the inhibition of MMP9 by increases in thrombospondin-1 (THBS1) and tissue inhibitor of metalloproteinase-1 (TIMP1) following bisphosphonate treatment. By inhibiting MMP9, MMP9-induced release of vascular endothelial growth factor (VEGF) from the extracellular matrix (ECM) is inhibited reducing VEGF-induction of angiogenesis. This then becomes a bisphosphonate-specific mechanism that inhibits angiogenesis and induces MRONJ.

Methods: Using RT-PCR arrays, we screened RNA isolated from the jaws of MRONJ, control, and ZA-treated rats 3 weeks after first molar extraction.

Results: Our study shows that *THBS1*, *TIMP1, and NRP2* mRNA are expressed in the tissues of the jaw in our rat model of MRONJ but not in control rats or rats treated with ZA alone without tooth extraction. The significance of this finding is that THBS1 and TIMP1 are known to inhibit neovascularization in certain cancers through the inhibition of MMP9. An additional finding in this study is an increase in Neuropilin2 (NRP2), a coreceptor of the VEGFr2 receptor. The decrease in angiogenesis stimulates the formation of NRP2 as a possible reactionary mechanism to promote VEGF binding in response to ischemia.

Conclusion: The production of *THBS1, TIMP1, and NRP2* mRNA in the jaws of animals with MRONJ suggests that they are part of a bisphosphonate-induced inhibition of angiogenesis after trauma that promotes the ischemia that ultimately leads to osteonecrosis.

Introduction

Bisphosphonates such as zolendronic acid (ZA) are powerful compounds used to treat osteoporosis and the complications of bone cancer metastasis. Bisphosphonates are known to improve bone density by direct inhibition of osteoclastic bone resorption. In a subgroup of individuals on bisphosphonate therapy, medication-related osteonecrosis of the jaw (MRONJ) or bone death of the jaws is a tragic side effect (Figures 1 & 2). MRONJ is also characterized by loss of blood supply to the jaws which may be an antecedent and cause of the bone death.[1,2]

Under normal activities, bones develop microcracks which the process of bone turnover or remodeling repairs. In the presence of bisphosphonate, the normal process of turnover in the bones of the mandible and maxilla is inhibited. As a result, bones increase in density but over time may become weak and prone to fracture.[3–5]

In addition to inhibiting bone turnover through a direct effect on osteoclasts, bisphosphonates are also known to have direct effects on blood vessels.[1,6] The mechanisms of these blood vessel effects work through alterations in endothelial cell migration, proliferation, survival, and apoptosis and by antagonizing the activity of vascular endothelia growth factor (VEGF).[7,8] These effects can produce bone that is susceptible to infections and can inhibit angiogenesis, an important mechanism in the bone healing process.[9]

Thrombospondin-1 (THBS1) is a 142 kDa glycoprotein that has been known for many years to be a potent inhibitor of angiogenesis that works through a pathway involving the inhibition of VEGF.[10] THBS1 has also been shown to deactivate MMP9, an enzyme required for the release of VEGF from the extracellular matrix.

Tissue inhibitor of matrix metalloproteinase-1 (TIMP1) is an inhibitor of several enzymes including MMP9.[11] TIMP1 interferes with MMP9 production by blocking the conversion of pro-MMP9 to the active MMP9 enzyme, with the same ultimate effect as THBS1 in inhibiting the release of VEGF from the extracellular matrix.

Neuropilin-2 (NRP2) is a coreceptor of VEGFr2. The mRNA for this

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molecule is also upregulated in MRONJ as a possible cellular response to the ischemia caused by the decrease in angiogenesis.[12] The present study explores the hypothesis that the inhibition of angiogenesis by THBS1 and/or TIMP1 is part of a MRONJ-specific pathway induced by bisphosphonates. This decrease in MMP9 inhibits VEGF release from the extracellular matrix, and thereby decreases VEGF binding to its receptor. This decrease in VEGF binding leads to a decrease in angiogenesis, tissue ischemia, and promotes bone cell death.

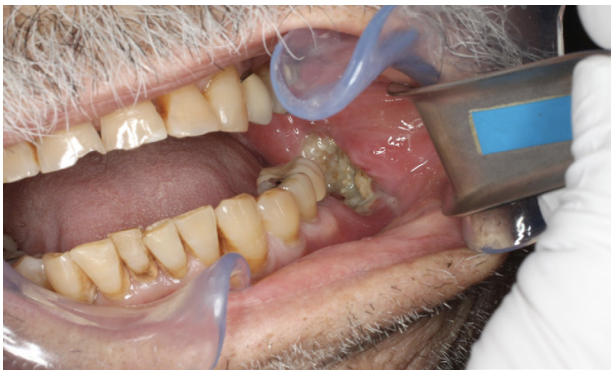

Figure 1 Exposed necrotic bone in a patient with MRONJ.

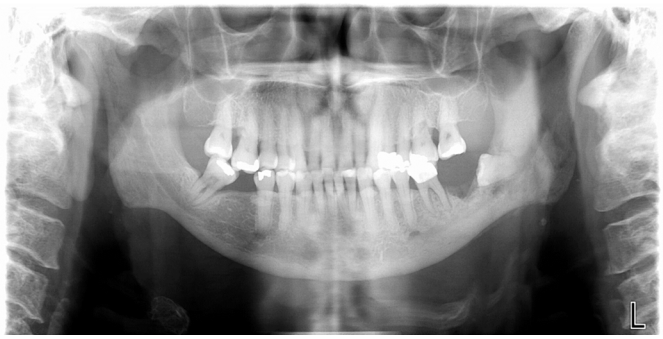

Figure 2 Radiograph of a patient with MRONJ showing bone loss in the mandible and maxilla.

Methods

Experimental model

The Institutional Animal Care and Use Committee of Western University of Health Sciences, Pomona CA reviewed and approved the experimental protocol used in this study which was adapted from a previous study by Marino *et al*.[13] Briefly, six Sprague-Dawley adult rats (Harlan, Indianapolis, IN, USA), weighing approximately 200g were purchased and provided with food and water *ad libitum* throughout the study. One week after arrival, four rats were injected with zoledronic acid (6µg/ 10µL/100g rat weight IV based on a human dose of 4mg/65.8Kg body weight). The additional two rats were injected with an equal volume of saline. The rats were divided into three groups. The saline-injected group was labeled "Control", two of the zoledronic acid-injected rats were labeled "ZA", and the other two zoledronic acid-injected rats were labeled as "MRONJ". Prior to zoledronic and saline injections, all rats were anesthetized with a rodent cocktail consisting of ketamine (100mg/mL), xylazine (20mg/ML) and acepromazine (10mg/mL).

After the onset of deep anesthesia, the right maxillary first molar was extracted from each of the two rats in the MRONJ group only. Three weeks after the first injection, the animals were re-anesthetized. All animals from each group (Control, ZA, and MRONJ) were sacrificed and the bone tissue harvested as described below. Rats in these groups were labeled 3-week Control; 3-week ZA; or 3-week MRONJ respectively.

On the day of sacrifice (week 3), maxillary bone tissue was harvested from the area adjacent to the first molar (Control and ZA groups) or first molar extraction site (MRONJ group) and frozen in liquid nitrogen before storage at -80ºC.

RNA Isolation

Frozen bone samples were placed into 1mL of TriReagent (Qiagen), prechilled in liquid nitrogen. Bone tissue was disrupted with a Polytron homogenizer at maximum speed for 45 seconds on ice. Solubilized bone extract was isolated from bone fragments by centrifugation at room temperature for 15 sec at 8,600 X g. RNA was purified from this bone extract using the RNeasy Plus Universal Mini protocol following manufacturer instructions (Qiagen. Valencia, CA). The amount of RNA present in each sample was determined using a NanoDrop® spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE).

RT-PCR

Experimental RNA samples were converted into first-strand cDNA using the RT^2 First Strand Kit (Qiagen Inc.-USA Germantown, MD). The cDNA was mixed with RT^2 SYBR Green qPCR Mastermix. This mixture was aliquoted into the wells of the RT^2 Profiler PCR Arrays for "Rat Angiogenic Growth Factors". PCR was performed using the ABI Step One Plus Real Time PCR System. Relative expression was determined using data from the real-time cycler and the ΔΔCT method.

Results and discussion

Heat maps constructed from Control, ZA-treated, and MRONJ rats show 3 genes of the 84 genes in the "Rat Angiogenic Growth Factors" array with increased expression in MRONJ rats that did not show increased expression in ZA-treated or Control rats after 3 weeks (Figure 3). These genes were *NRP2*, *THBS1* and *TIMP1*. The neuropilin-2 or *NRP2* gene codes for a co-receptor for the VEGFR2 receptor. The thrombospondin-1 or *THBS1* gene codes for an anti-angiogenic factor that interferes with MMP9-mediated release of VEGF from the extracellular matrix. The tissue- inhibitor of matrix metalloproteinase-1 or *TIMP1* gene codes for an inhibitor of multiple MMPs and is known to complex with pro-MMP9.

In our rat model, bisphosphonate alone does not readily induce MRONJ. This is because the need for robust angiogenesis is not as great when the tissue is intact. The inhibition of angiogenesis is far more apparent when the tissue is trying to repair an open wound like that seen after a dental extraction. In the proposed THBS1/TIMP1 anti-angiogenic pathway (Figure 4), this would also be expected to be the case. This mechanism describes the inhibition of the compensatory VEGF and angiogenic pathway necessary to repair damage at the extraction site.

The proposed THBS1/TIMP1 anti-angiogenic pathway is supported by the findings from other studies where the loss of VEGF results in the interruption of vascular development.[14] In addition, endothelial cell migration is important to the formation of new capillaries, and THBS1 antagonize this process.[15] It was previously known that THBS1 inhibits the release of VEGF from the extracellular matrix through suppression of MMP activity.[16] The level of active MMP9 has

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also been shown to be inversely correlated with the level of THBS1 in certain cancers.[17]

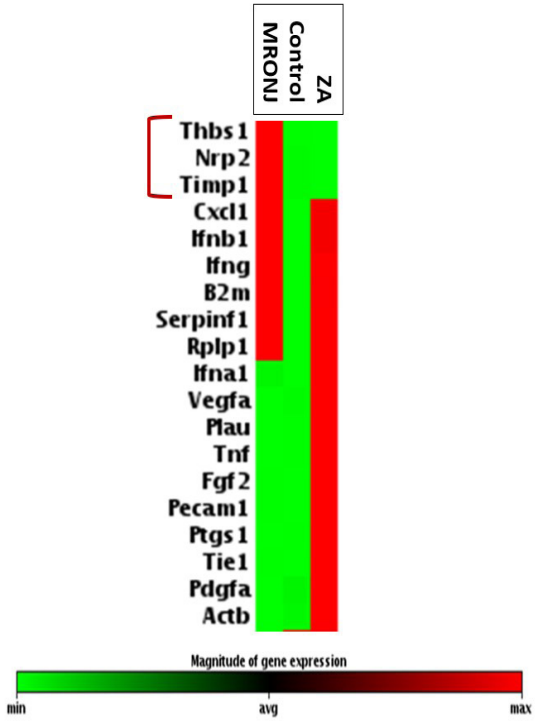

Figure 3 Portion of a heat map from the 3-week groups showing maximum expression in THBS1, NRP2, and TIMP1 in the MRONJ but not the Control or Zolendronic acid treated rats.

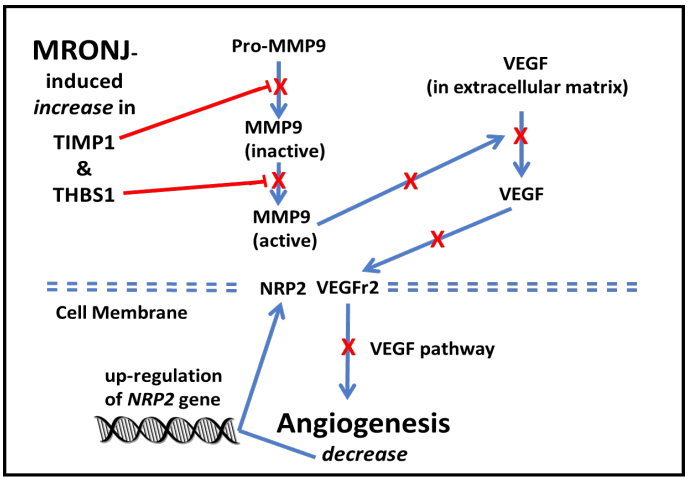

Figure 4 Proposed pathway for MRONJ-induced inhibition of angiogenesis involving THBS1 and TIMP1 decrease in MMP9 release of VEGF from the extracellular matrix.

The finding of an increase in NRP2 is also supported by previous studies showing that VEGF activity is enhanced by VEGF co-receptors, such as NRP2.[18] In ischemia, a mechanism involving the upregulation of VEGFr2 co-receptor NRP2 would be expected. Taken together, previous studies by others and our results support a role for the THBS1/TIMP1 inhibition of angiogenesis through MMP9 as a mechanism in oral tissue injury that would lead to slow wound healing and the development of MRONJ.

Ackowledgments

The Work is supported by a grant from the Western University of Health Sciences Office of Research and Biotechnology

Conflicts of interest

The authors declare no conflicts of interest.

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