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Changes in mandibular CDH5, CXCL1, and PECAM1 expression following exposure to bisphosphonate and molar extraction suggest a loss of vascular endothelial cell barrier integrity plays a role in MRONJ

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ABSTRACT

Objectives: Medication related osteonecrosis of the jaw (MRONJ) is a disorder characterized by loss of blood supply to the jaws and death to the bone. In our previous work, we created a rat model of MRONJ by multiple injections of 60ug/Kg zoledronic acid (ZA) via tail vein followed by extraction of a single first molar. We have previously shown in this model a decrease in the vasculature of the jaws and a delay in bone healing after 6 weeks. The current study was designed to look at mRNA expression and immunohistochemical localization of three endothelial cell markers (CDH5, CXCL1 and PECAM1) 6 weeks following ZA injection with and without molar extraction and in saline injected rats as controls. The objective of this study is to determine if the expression of these markers may serve to identify clinically significant changes in vascular endothelium barrier function associated with the onset of MRONJ.

Methods: Using RT-PCR, we analyzed the expression of mRNA for angiogenic markers, six weeks after injection with either 60ug/Kg ZA or saline as a control and in ZA injected rats following first molar extraction. Routine immunohistochemical procedures with antibodies specific for rat marker proteins were used to study the immunohistochemical localization of proteins translated from the mRNA angiogenic markers with significant expression. All tissues were processed together under identical conditions.

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Results: We found a decrease in the mRNA expression of CDH5, and PECAM1 and an increase in CXCL1 expression six weeks after extraction and ZA injection in our rat model of MRONJ. Rat monoclonal antibodies specific for protein epitopes expressed by these genes (*VE-cadherin*, *C-X-C motif ligand 1(CXCl1)*, and *PECAM-1*) were then used to localize proteins for these markers in tissues from ZA, saline-injected control rats, and MRONJ rats. The results show the absence of immunohistochemical localization of *PECAM-1* and *VE-cadherin* marker proteins (-) in tissues from the MRONJ rats but strong (++++) in tissue from the saline-injected Control rats. The results also show the localization of the immunohistochemical staining for *CXCl1* was high (++++) in the MRONJ rats after six weeks but absent (-) in the Controls. No mRNA or immunohistochemical localization for the three markers was seen in the rats treated with ZA without extraction.

Conclusions: Our study identifies markers associated with a loss of vascular endothelial cell barrier integrity suggesting a role for this mechanism in MRONJ. This finding further suggests that CDH5, CXCL1 and PECAM1 may be useful as early markers for the changes seen in blood vessels after bisphosphonate exposure but before the frank onset of MRONJ.

Keywords and abbreviations: bisphosphonate; osteonecrosis; medication related osteonecrosis of the jaw (MRONJ); zoledronic acid (ZA).

Introduction

Medication related osteonecrosis of the jaw (MRONJ) is a disorder characterized by loss of blood supply to the jaws and death to the bone. In our previous work, we created a rat model of MRONJ by multiple injections of 60ug/Kg zoledronic acid (ZA) via tail vein followed by extraction of a single first molar [1]. We have previously shown in this model a decrease in the vasculature of the jaws and a delay in bone healing after 6 weeks [2]. The current study was designed to look at mRNA expression for three marker genes (CDH5, CXCL1 and PECAM1) and immunohistochemical localization of the expressed proteins/peptides *VE-cadherin*, *C-X-C motif ligand 1 (CxCl1)* and *PECAM-1* at 6 weeks following ZA injection. The objective of this study is to determine if the expression of these markers may serve to identify clinically significant vascular changes occurring with the onset of MRONJ.

Materials and 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* [1]. Briefly, twelve 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, eight 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 four rats were injected with an equal volume of saline. The rats were divided into

three groups. The saline-injected group was labeled “Control”, four of the zoledronic acid-injected rats were labeled “ZA”, and the other four 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 mandibular first molar was extracted from each of the four rats in the MRONJ group only. Three weeks after the first injection, all twelve animals were re-anesthetized and re-injected. Three weeks after the second injection, all animals in each group (Control, ZA, and MRONJ) were sacrificed and the bone tissue harvested as described below. Rats in these groups were labeled 6-week Control; 6-week ZA; or 6-week MRONJ respectively.

On the day of sacrifice, mandibular bone tissue was harvested from the area adjacent to the first molar or first molar extraction site in each group and frozen in liquid nitrogen before storage at -80^{o}C. Tissue from each mandible was also harvested and placed in phosphate-buffered formalin before decalcification in EDTA and paraffin embedding for histology and immunohistochemistry.

RNA Isolation:

Frozen bone samples were placed into 1mL of TriReagent (Qiagen), prechilled in liquid nitrogen. Bone tissue was disrupted with a Polytron® homogenizer (Kinematica, Bohemia, NY) 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 $\Delta\Delta$CT method.

Immunohistochemistry:

All tissues were decalcified in EDTA and fixed in a 10% phosphate buffered formalin solution before dehydration in a graded series of ethanol and xylene washes. Decalcified tissues were then embedded in paraffin. Sections of 5um thickness were cut and placed on glass slides.

These tissue sections for localization studies were processed using the avidin-biotin procedure. Briefly, the tissues were deparaffinized in D-limonene (Sigma Chemical Co.) for 5min. For this procedure the tissue sections were rehydrated with two washes of 100% ethanol, followed by 95% ethanol, and two washes of phosphate-buffered saline, pH 7.4 (PBS), for 3 min each. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. Nonspecific antibody binding was also blocked by exposure of the section to a 1:75 dilution of normal horse serum for 20 min (Vector Laboratories, Inc., Burlingame, CA). The sections are then incubated for 30 min in a humid chamber with a 1:500 dilution of mouse anti-rat antibodies to the target proteins (anti-*VE-cadherin*, anti-*PECAM-1* and anti- *CxCl-1*). Tissues incubated in normal mouse serum were used as negative controls. After rinsing in PBS, the sections were incubated for 30 min in a 1:200 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, Inc.). The sections were again rinsed and incubated for 30 min in a solution containing 45ul of avidin-peroxidase solution A, 45ul of avidin-peroxidase solution B, and 5 ml of PBS (Vector Laboratories, Inc.).

Sections were rinsed thoroughly in PBS and incubated for 10 min in a PBS solution containing 0.05M 3,3'-diaminobenzidine tetrahydrochloride grade II (Sigma Chemical Co.) plus 0.03% hydrogen peroxide. After 10 min, the slides were rinsed in water, counterstained for 5 min with Mayer's hematoxylin, dipped in ammonia water and rinsed for 10 min. Tissue sections were then dehydrated in ethanol and xylene and cover slipped with mounting media before photographing.

Results

A heat map of the mRNA data (Figure 1) shows an increase in CXCL1 mRNA expression in the MRONJ rat model relative to Control rats and ZA-treated rats without molar extraction. By contrast our mRNA data shows that high levels of CDH5 and PECAM1 in Control rats that disappears in rats after exposure to zoledronic acid (ZA rats). It is noted that PECAM1 mRNA expression returns in the MRONJ rats.

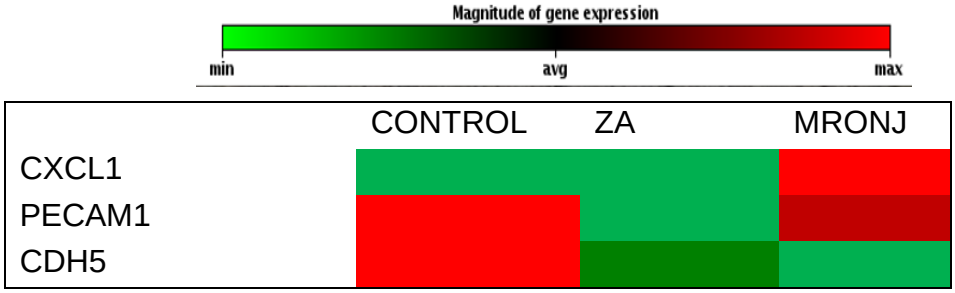

Figure 1: Heat map summary of mRNA expression after 6-weeks.

Results of the immunohistochemical localization studies (Figure 2-5) show localization of *CxCl1* protein in blood vessels of MRONJ rat mandibles only (Figure 3). Localization of *PECAM-1* (Figure 4) and *VE-cadherin* (Figure 5) were also shown to be associated with blood vessels but only in Control rats that had not been exposed to either extraction and/or zoledronic acid.

	CONTROL	ZA	MRONJ
CxCl1 1	(-)	(-)	(+++)
PECAM-1	(+++)	(-)	(-)
VE-cadherin	(++++)	(-)	(-)

Figure 2: Immunohistochemical localization summary of results.
Scale: (-) no localization to (++++) maximal localization.

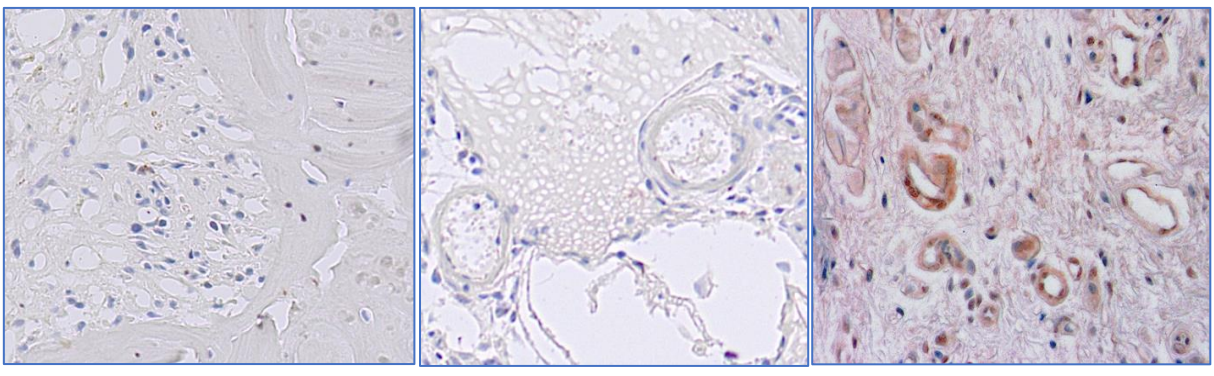

Figure 3. Immunohistochemical localization of *CxCl 1* in rat mandibular blood vessels (40x): A) Control rat (-), B) ZA-treated rat (-), C) MRONJ rat-ZA-treated plus extraction (++++)

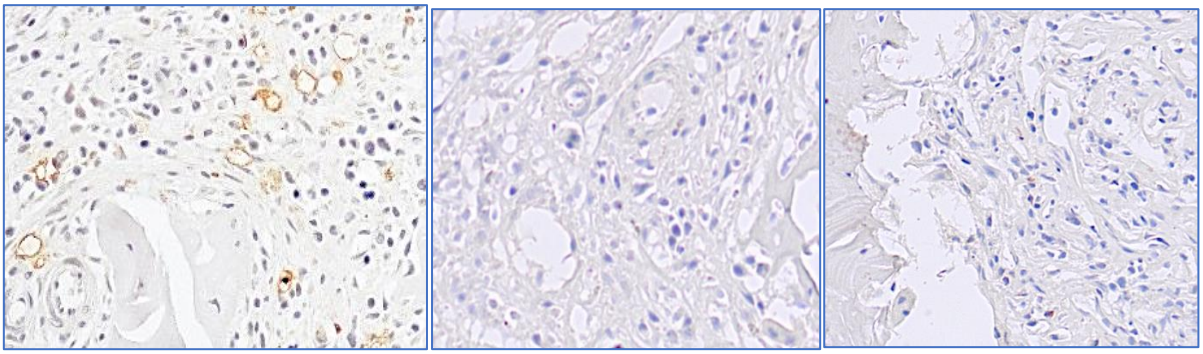

Figure 4. Immunohistochemical localization of *PECAM 1* in rat mandibular blood vessels (40x): A) Control rat (+++), B) ZA-treated rat (-), C) MRONJ rat-ZA-treated plus extraction (-)

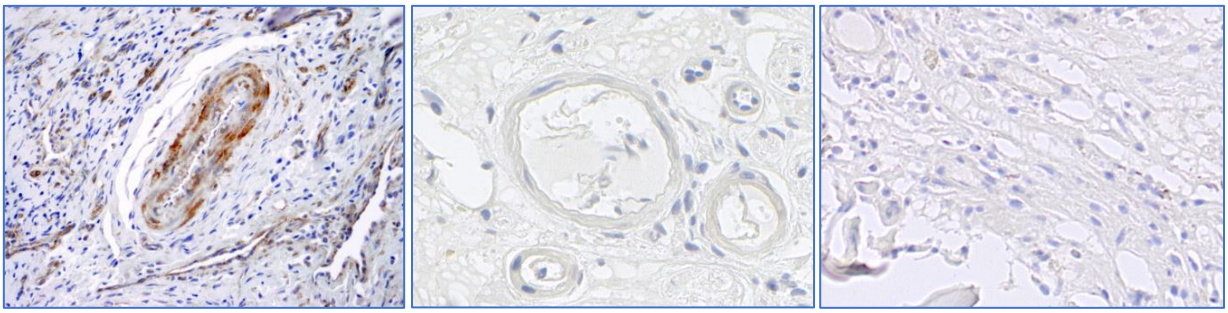

Figure 5. Immunohistochemical localization of *VE-Cadherin* in rat mandibular blood vessels (40x): A) Control rat (+++), B) ZA-treated rat (-), C) MRONJ rat-ZA-treated plus extraction (-)

Discussion

It has been established previously that an increase in shear stress on the vascular endothelium increases the expression of *CxCl1*, a molecule on neutrophils that plays a role in rolling and adhesion of neutrophils to the endothelium [3]. CxCL1 also promotes arteriogenesis through a mechanism involving monocyte recruitment [4]. In our study we observed an increase in CXCl1 mRNA and *CxCl1* peptide in tissues from our rat model of MRONJ which supports this.

Our study also shows a decrease in CDH5 mRNA and *VE-cadherin* protein associated with MRONJ. *VE-cadherin* is a cadherin from the cadherin superfamily and plays an important role in endothelial cells through control of the cohesion and organization of intercellular junctions [5,6]. Integrity of intercellular junctions is a major determinant of permeability of the endothelium, and the *VE-cadherin*-based adherens junction is thought to be particularly important. *VE-cadherin* is known to be required for maintaining a restrictive endothelial barrier [5,6]. The loss of expression of this molecule is also in keeping with our hypothesis that a loss of vascular endothelial cell barrier integrity plays a role in MRONJ.

VE-cadherin is also indispensable for proper vascular development, as demonstrated in studies with transgenic mouse models of CDH5 deficiency [6]. These models were embryonic lethal due to vascular defects. Further studies using one of these models revealed that although vasculogenesis occurred, nascent vessels collapsed or disassembled in the absence of CDH5 [7]. Therefore, it was concluded that expression of CDH5 served the purpose of maintaining newly formed vessels in their mouse model. The loss of CDH5 expression in our rat model of MRONJ supports their work.

We also demonstrated mRNA expression of PECAM1, in Control rats and in the MRONJ rat model. *PECAM1* protein expression was also found in Control rats but not found in ZA-rats or

MRONJ by immunohistochemistry. *PECAM1* is generally found in both the neutrophils and the endothelium which when bound to each other stimulate dismantling of the *VE-cadherin* adhesion molecules and opening the epithelium to diapedesis (see figure 6) [8,9].

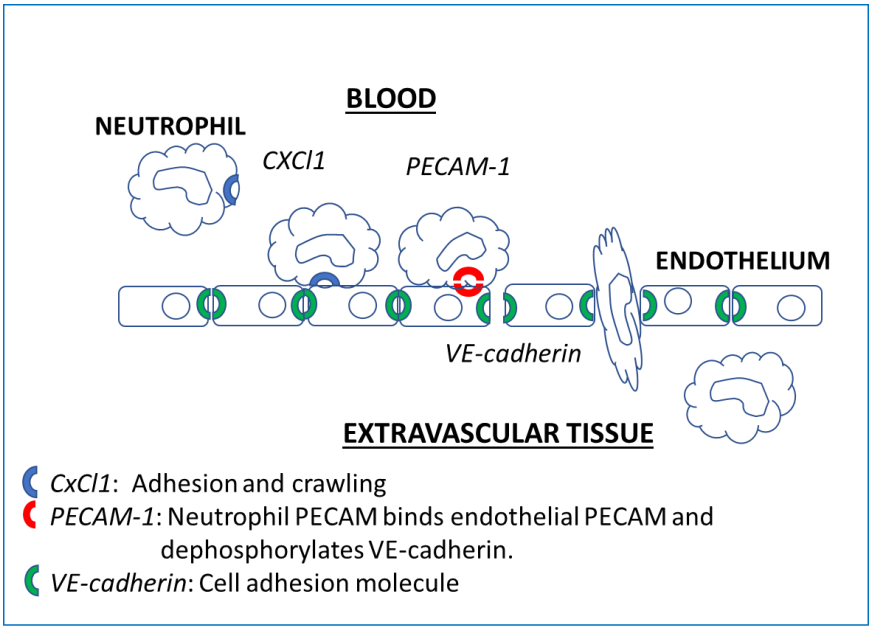

Figure 6: Extravasation mechanism involving *CxCl1, PECAM-1 and VE-cadherin* that may function as a pathway for transmigration of the immune system through endothelial cells as part of the mechanism for the damage seen in MRONJ.

One of the normal actions of *VE-cadherin* in maintaining blood vessel integrity is to block leukocyte extravasation [6]. Our data suggests that in MRONJ rats, with a drop in the level of *VE-cadherin*, the level of *PECAM1* increases and macrophages arrive and adhere at the site of injury [8]. These macrophages secrete factors that include *CxCl1* [10]. CXCL1 mRNA and *CxCl1* protein are expressed at high levels during inflammatory responses thus contributing to the process of inflammation. *CxCl1* is also involved in the process of wound healing. *CXCl1* can also activate T-helper cells [11]. T-cells are major components of the adaptive immune system that assist the activity of other immune cells by releasing cytokines that alter the behavior of the target. Their roles include directly killing infected host cells, activating other immune cells, and regulating the immune response. In our study *CXCl1* increases in the MRONJ rats relative to the Control rats and the ZA rats without extraction.

In conclusion, our study supports a role for disruption of endothelial integrity in the pathogenesis of MRONJ by a mechanism involving a drop in the expression of mRNA and protein for the CDH5 and PECAM1 genes and an increase in expression of CXCL1.

Acknowledgement

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