Altered VEGF Regulation in Ovariectomized Rat Alveolar Bone Sockets by Bisphosphonate

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Bisphosphonates reduce bone loss by suppressing osteoclast activity and are currently used for the treatment of osteoporosis. As a side effect of bisphosphonates treatment, the emergence of osteonecrosis of the jaw (ONJ) has stimulated interest in elucidating how bisphosphonates affect the bone, but far less understanding exists as to the ONJ mechanism. In the present study, it was hypothesized that bisphosphonates may affect the healing of the alveolar socket by altering VEFG regulation. *In vivo* osteoporosis rat models were made by bilateral surgical ovariectomy. Pamidronate (3.5 mg/kg/wk) was IP injected for 8 wks and the maxillary 1st molars were bilaterally extracted. For *in vitro* test, periodontal ligament cells were treated with pamidronate. Unexpectedly, VEFG-A and -B mRNA and protein levels were increased in *in vivo* and *in vitro* by the high concentration treatment of bisphosphonates. However, VEGF-C level was not changed. In the socket at day 7 after the molar extraction, the socket was filled with infiltration of neutrophils which showed strong immunoreactivity against VEGF. VEGF-B mRNA was in particular upregulated by the pamidronate treatment. This result suggest that high dose pamidronate may alter VEGF expression that may serve as another mechanism of ONJ.

Keywords: VEGF, Bisphosphonates, ONJ

Introduction

Bisphosphonates are widely used for the prevention and treatment of osteoporosis, which is frequent found in postmenopausal women and older adults. These increase bone mass and bone trabecular thickness by inhibiting osteoclastic bone resorption. Bis-phosphonates are also used to treat multiple myeloma, bone metastasis, and Paget's disease. Although bisphosphonates are beneficial, side effects may occur during or even after treatment. The treatment of nitrogen-containing bisphosphonates may result in osteonecrosis of the jaw (ONJ), first identified since 2003 in individuals administered intravenous bisphosphonate therapy and in individuals receiving oral therapy since 2006[1]. ONJ involves necrotic exposed bone in the jaw, pain, possible secondary infection,

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swelling, painful lesions and various dysesthesias[2,3]. The Food and Drug Administration lists alendronate, etidronate, ibandronate, risedronate, pamidronate, zoledronic acid, zoledronate, and tiludronate as potentially increasing the risk of ONJ[4]. Seven to ten % of individuals with multiple myeloma and 4% of those with breast cancer had ONJ[2]. Reports of ONJ after oral therapy vary, but amount to 4% of bisphosphonate users[1,5,6].

Various theories to explain the high risk of ONJ in the mandible and maxilla include inhibition of osteoclasts, decrease of angiogenesis and modification of blood vessels[7] and suppression of bone turnover, all of which cause accumulation of microdamage of jaw bone[8]. This microdamage may eventually lead to microfractures with trauma and infection and localized ONJ may result if the bone is not cared sufficiently[7]. Secondary infection, commonly actinomycosis, will exacerbate the problem[9]. Retarded epithelial regeneration and diminished vascularity also have been implicated as pathogenic features of ONJ[10].

Angiogenesis, the development and remodeling of new blood vessels from an already existing vasculature, plays an important role in tissue growth and repair during wound healing. Moreover, angiogenesis is a fundamental regulatory process involved in the pathogenesis of several human diseases. The maintenance of vascular homeostasis is dependent upon the balance of pro- and antiangiogenic factors and is tightly regulated by receptor-ligand interactions as well as interactions between cells and the extracellular matrix[11,12]. The hallmark for healing of the alveolar socket after tooth extraction is the ability to develop a vascular supply since growth and repair of

tissues require nutrients, growth factors and oxygen which are mainly supplied by blood vessels. Thus, angiogenesis is a fundamental requirement for the socket healing.

Vascular endothelial growth factor (VEGF) is expressed temporospatially during angiogenesis *in vivo*. The VEGF family comprises six subgroups of proteins: VEGF-A, -B, -C, -D and -E and placenta growth factor (PlGF)[13]. VEGF-A is the most potent and pivotal mediator of angiogenesis and a highly specific mitogen for vascular endothelial cells. The VEGF-A gene is organized as a single gene of approximately 14 kbp. Differential splicing of full-length VEGF-A pre-mRNA gives rise to two known families of proteins consisting of multiple isoforms that differ by only six amino acids at their C-terminus. Thus far, at least 14 subtypes of VEGF-A are known: namely, VEGF111, VEGF121, VEGF121b, VEGF145, VEGF145b, VEGF148, VEGF162, VEGF165, VEGF165b, VEGF183, VEGF183b, VEGF189, VEGF189b and VEGF206. Conventional VEGF-Axxx isoform family is angiogenic, while the VEGF-Axxxb family is antiangiogenic[14,15]. VEGF-B can induce inflammatory cell chemotaxis and has a role to play in inflammatory angiogenesis[16]. VEGF-C is expressed during embryonic development in regions where lymph vessels sprout from blood vessels. In adults, VEGF-C is mainly restricted to the lymphatic endothelium and has been implicated in the development of lymphatic vessels[17,18].

Bisphosphonates are known to have antiangiogenic properties owing to their ability to significantly decrease circulating levels of VEGF[9,19]. Spite of the importance, there are few reports to elucidate the relationship between the expression of overall

VEGF isoforms and ONJ. In the present study, as a causative factor for ONJ, it was hypothesized that bisphosphonates inhibit angiogenesis and induce inflammatory reactions by regulating VEGF regulation. This study tested the hypothesis.

Materials and Methods

In vitro study
Cell viability test

SV1103 mouse periodontal ligament cell line was used in *in vitro* study. Cells were cultured in Dulbecco's Modified Eagle Media (Gibco-BRL, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, MD, USA) and 1% penicillin-strepto-mycin in a humidified atmosphere of 5% CO_2 at 37℃. Cell viability was tested using Cell proliferation and Cytotoxicity Assay kit (Dojindo, Tokyo, Japan). $2*10^4$ cells/well were seeded in a 96 well plate for 16 hours and thereafter, treated with disodium pamidro-nate (Hanlim pharmaceutical company, South Korea) at different concentrations for 1 and 3 days. Optical density was spectrophotometically measured at 450 nm using ELISA reader EL800 (BioTek, VT, USA).

mRNA and protein levels

Transcriptional and translational levels of VEGF isoforms were determined by RT-PCR and Western blotting, respectively. mRNA level was also determined by real time RT-PCR and RT-PCR and Western blotting was described below *in vivo* study.

In vivo study
Animals

All procedures were performed in accordance with the ethical standards formulated by the animal care and use committee in Chonnam National University. Seven week-old female Sprague-Dawley rats weighing approximately 200g were housed in laboratory animal care-approved facilities for 1 week for acclimatization.

Induction of osteoporosis and bisphosphonate treatment

For all experimental period, standard food of pellets was provided ad libitum with water. All rats were then randomly divided into two groups: control group (n = 15, osteoporosis group) and experimental group (n = 15, osteoporosis and bisphosphonate group). To establish standard osteoporosis animal model in the co ntrol[20], bilateral surgical ovariecto-my was performed and housed for 16 weeks. Briefly for ovariectomy, general anesthesia was achieved by intraperitoneal injections of Zoletil 50 (0.05 mg/kg, Virbac, Carros cedex, France). After abdominal region was disinfected with potadine and shaved, abdominal cavity was opened to surgically remove bilateral ovaries. All rats received intramuscular antibiotic injection for 3 postoperative days. For experimental group, the same procedures were performed for ovariectomy. At week 8 after ovariectomy, rats underwent intraperitoneal injection of disodium pamidronate (3.5 mg/kg/wk, Hanlim pharmaceutical company, Seoul, South Korea) for further 8 weeks to establish osteoporosis prevention model. At week 17 after ovariectomy, the first maxillary molars were ex-tracted bilaterally under anesthesia without causing

fractures in the alveolar bone.

Histological preparations

Maxillary alveolar sockets for the 1st molars were surgically isolated at day 3 and 7 after extraction and immersion-fixed in 4% paraformaldehyde solution overnight. Tissues were decalcified in ethylene diamine tetra-acetic acid (pH 7.4) over several weeks and were then routinely processed for embedding in paraffin. Sagittal sections were cut 5 μm-thick for H-E and immunofluorescence staining.

RNA preparation

After portions of the alveolar bone for the 1st maxillary molars at day 3 and 7 after extraction were surgically cut, they were immediately frozen in liquid nitrogen and crushed as finely as much. Total RNA was extracted using a Trizol® Reagent (Invitrogen, Oregon, USA) and RNA samples were treated with DNAse I (Gibco BRL, MD, USA). RNA samples were quantitated using Nonodrop 2000 spectrophotometer (Thermo scientific, DE, USA) and qualified by measuring OD 260/280 ratios.

RT-PCR

Primers were custom-designed. The housekeeping gene b-actin was used as a reference. Their base sequences, amplicon size and Genbank No. were summarized in Table 1. For the reverse transcription reaction and PCR, AccPower® RT PreMix (Bioneer, Daejeon, Korea) and AccPower® PCR PreMix (Bioneer, Daejeon, Korea) was used, respectively. Briefly, mixtures of total RNA and 200 pmole Oligo dT$_{18}$ were incubated at 70℃ for 5 min and transferred to AccPower RT PreMix tube. cDNA synthesis reaction

was performed at 42℃ for 1 hr, followed by incubation at 94℃ for 5 min for the inactivation of the reverse transcriptase. PCR reactions were conducted in a Palm-Cycler thermocycler (Corbett Life Science, Sydney, Australia) with the following profile: denaturation for 30 sec at 95℃, annealing for 1 min at 60℃ and 30 sec extension step at 72℃, followed by a final extension step of 10 min at 72℃. Products were resolved on a 1.5% agarose gel and stained with SYBG® safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). Products were resolved on 1.2% agarose gel and stained with SYBG® safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and their product size was checked using a 100 bp marker (Takara, Otsu, Shiga, Japan). For the negative PCR control, cDNA template was substituted with distilled water.

Real-time RT-PCR

cDNA synthesis was performed as described in the RT-PCR section and used for the real-time amplification of the target genes. Amplified cDNA was detected using the SYBR Green PCR Master Mix Reagent kit (Qiagen, Valencia, CA, USA). PCR conditions were as follows: incubation for 10 min at 95℃, followed by 27 cycles of 10 sec denaturation at 95℃, annealing for 15 sec at 63℃ and 20 sec extension at 72℃. The target and reference genes were amplified in separate wells using a Rotor-Gene RG-3000 (Corbett Research, Morklake, Australia). The reaction mixture lacking cDNA was used as a negative control in each run.

Data were analyzed using the Corbett Robotics Rotorgene software (Rotorgene 6 version 6.1, Build 90 software). Ratios of the intensities of the target genes and b-actin signals were used as a relative

measurement of the expression level of target genes. To ensure experiment accuracy, real-time PCR assays were conducted in triplicate for each sample. The mean fold change of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and the range of the fold changes was calculated from the standard error of the $\Delta\Delta Ct$ values.

Western blot

Protein was extracted from portions of the alveolar bone for the 1st maxillary molars at day 3 and 7 after tooth extraction using Ready prep protein extraction kit (Bio-RAD, Hercules, CA, USA). After samples were crushed in a liquid nitrogen solution and resolved in the protein extraction solution with a proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 1 hr, supernatant protein lysates were quantified using Amersham GeneQuant Pro (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). After boiled in a 5x SDS sample buffer for 10 min, protein lysates were loaded onto 10% continuous gradient SDS-polyacrylamide gel and then

Table 1. Oligonucleotides used for RT-PCR

Gene	Sequence (5' to 3')	Amplicon (bp)	Genbank No.
mVEGF-A	Forward TCCTCTCCTTACCCCACCTC Reverse TCTTTTCTCTGCCTCCGTGA	190	NM_001110268.1
mVEGF-B	Forward ACGGGCCCGGCTGCTTTTATG Reverse TGAGGGGAGACAGCCAGCCA	259	NM_001185164.1
mVEGF-C	Forward AGAAGTATGCCGCTGTGTCC Reverse TGTTACCATGGTCCCACAGA	152	NM_009506.2
mβ-actin	Forward GAATCCTGTGGCATCCATGA Reverse TCAGCAATGCCTGGGTACAT	138	NM_007393.2
rVEGF-A	Forward GCAGATCATGCGGATCAAAC Reverse ACGCGAGTCTGTGTTTTTGC	202	BC_168708
rVEGF-B	Forward AGACTTTTTCCACGGGCTTT Reverse TGCATTTCCTTTTTGGAACC	201	NM_053549.1
rVEGF-C	Forward CGCTGTGTCCCATCATATTG Reverse GCCAGTCCATTCCCACAGTA	197	NM_053653.1
rβ-actin	Forward GCTGACAGGATGCAGAAGGA Reverse TGGACAGTGAGGCCAGGATA	124	NM_031144
m VEGF A-165	Forward GTCCAAGATCCGCAGACGTGTAA Reverse CTCACCGCCTTGGCTTGTCACAT	182	NM_001025250.3
m VEGF A-165b	Forward GTCCAAGATCCGCAGACGTGTAA Reverse TAATCGGTCTTTCCGGTGAGAGGT	115	NM_001025250.3

r: rat, m: mouse

transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked with TBS-T buffer [10 mM Tris-buffered isotonic saline (pH 7.0), 0.1% merthiolate, 0.1% Tween-20] containing 5% skim milk for 1 hr at room temperature with shaking. The membrane was then incubated with 1: 1000 purified rabbit polyclonal primary antibody raised against amino acids of VEGF (Abcam plc, Cambridge, UK) overnight at 4℃ with gentle shaking. Purified mouse monoclonal primary antibody to β-actin (Sigma-Aldrich Co., ST Louis, MO, USA) was used for the reference. Washed with TBST for 10 min, the membrane was incubated with 1:3000 anti-rabbit horseradish peroxidase (HRP)-conjugated antibody for VEGF and anti-mouse HRP-conjugated secondary antibody for β-actin (Cell Signaling Technology, Beverly, MA, USA) for 2 hr. Bound antibodies were visualized with Immobilon Western chemiluminescent HRP substrate (Millipore, Billericas, MA , USA).

Immunofluorescence stain

Tissue sections were routinely processed and rinsed with PBS. The same primary antibody of 1:500 dilution against VEGF which was used for the Western blot were used for the immunofluorescence stain.

Immunofluorescence staining was performed using TSA™ Kit (Invitrogen, Carlsbad, CA, USA). Briefly, after endogenous peroxidase in sections was blocked by 1% H_2O_2 for 1 hr, they were reacted with primary antibodies overnight, followed by incubation in HRP-conjugated secondary antibody for 1 hr. Finally, they were then incubated in Alexa Fluor 488® tyramide working solution for 10 min, followed by

propidium iodide stain for nuclei. Reactants were visualized and photographed using an LSM confocal microscope (Carl Zeiss, Standort Göttingen-Vertrieb, Deutschland). The primary antibody was substituted with normal serum for testing immunological specificity.

Results

In vitro
Cell viability test

SV1103 mouse periodontal ligament cells were tested for their viability after the pamidronate treatment for 1 and 3 days. By the treatment at a concentration 20 µg/ml only for 1 day, absorbance value was significantly reduced. By the treatment for 3 days, absorbance value was significantly reduced at a concentration 10 and 20 µg/ml (Fig. 1).
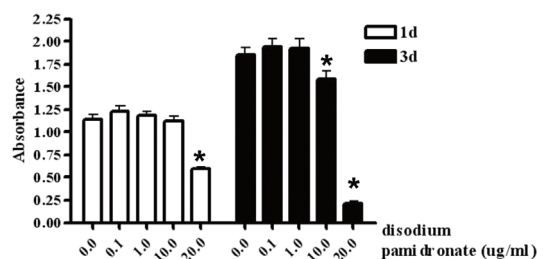
Figure 1. SV1103 mouse periodontal ligament cells were treated with pamidronate for 1 and 3 days. Absorbance value was significantly reduced by the treatment at 10 µg/ml concentration for 3 days and 20 µg/ml concentration for 1 and 3 days. Data represent means ± SE of measurements (n = 5). Each value obtained from pamidronate treatment was compared with the control (0 µg/ml). * p < 0.05

Effects on mRNA and protein levels

SV1103 mouse periodontal ligament cells were tested for changes in Vegf mRNA and protein expression after the pamidronate treatment at concentration of 0.1 μg/ml to 10 μg/ml for 1 and 3 days. Western blot results revealed that the protein level was increased by the 10 μg/ml treatment for 3 days (Fig. 2a). To further analyze changes in each VEGF isoform, mRNA levels of VEGF-A, -B and -C were determined, revealing that VEGF-A were increased approximately 5 and 13 times, respectively and VEGF-B were also increased 3 and 17 times by the 10 μg/ml treatment for 1 and 3 days, respectively. VEGF-C mRNA level was increased, but the fold change was negligible (Fig. 2b). VEGF-A is the most potent mediator of neovascularization in both health and disease. In the present study, effects of pamidronate treatment on VEFG-A$_{165}$ and VEFG-A$_{165}$b were investigated. Both VEFG-A$_{165}$ and VEFG-A$_{165}$b were increased significantly (Fig. 2c).

In vivo

Effects on mRNA and protein levels

To investigate the effects of bisphosphonate on VEGF, pamidronate (3.5 mg/kg/wk) was treated for 3 and 7 days. Alveolar bone for the maxillary 1st molars including remnants of periodontal ligaments after tooth extraction were surgically isolated for the extraction of proteins and RNA. Western blot result revealed that VEGF protein level was apparently increased by the pamidronate treatment for 3 days after ovariectomy (Fig. 3a). These results were also confirmed at a transcription level by RT-PCR. The VEGF-A and -B mRNA level was increased by the pamidronate treatment for 3 and 7 days after ovariec-

tomy, respectively. However, VEGF-C mRNA level was not affected (Fig. 3b).
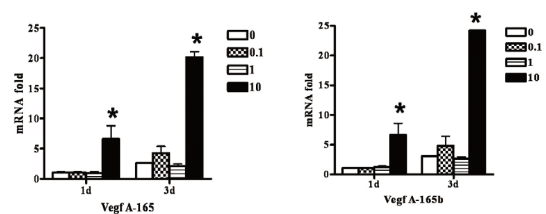
(a)
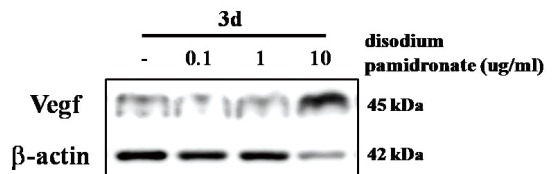
(b)
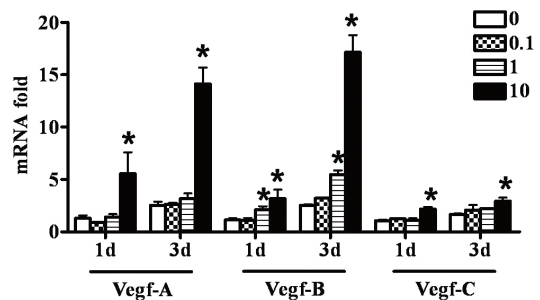
(c)

Figure 2. (a) Western blot results revealed that the VEGF protein level was much increased by the 10 μg/ml treatment for 3 days. (b) The gel image from RT-PCR revealed that amplicons for VEGF-A, -B and -C isoforms were generated and also confirmed quantitatively by real time RT-PCR. (c) Real time RT-PCR result revealed that both proangiogenic VEGF-A 165 and antiangiogenic VEGF-A 165b were increased approximately 7 times by the 10 μg/ml treatment for 1 day and this increase was enhanced by the treatment for 3 days. Real time RT-PCR data (means ± SE) were from 3 independent experiments. The comparison was made within the same day and isoforms. * $p < 0.05$.

(a)

(b)
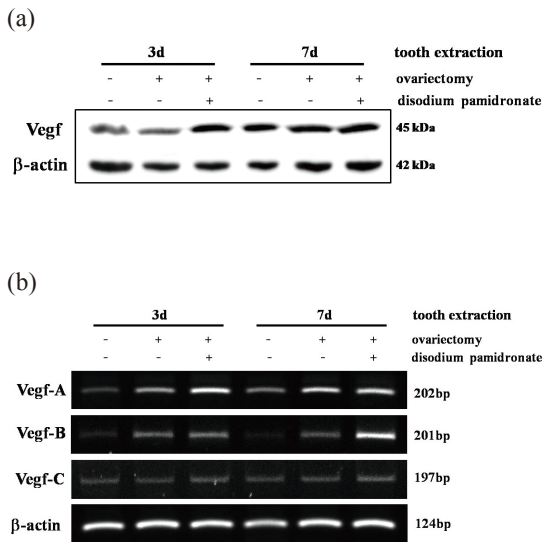
Figure 3. (a) Western blotting also revealed that 45 kDa protein of VEGF were increased by the pamidronate treatment for 3 days after ovariectomy. (b) The gel image of RT-PCR obtained from *in vivo* samples which were obtained from the alveolar socket after tooth extraction. The transcriptional level was increased by the pamidronate treatment for 3 days after ovariectomy.

H-E and Immunofluorescence staining

Morphological changes after tooth extraction were observed by H-E staining. The alveolar socket of rats without ovariectomy and pamidronate treatment at day 7 after tooth extraction was on the healing process with immature woven bone and connective tissues. Also, epithelium and lamina propria tissues were continuous and covering the growing bony tissues. The alveolar socket of ovariectomized rats without pamidronate treatment was largely filled with connective tissues. From the peripheral region of the alveolar socket woven bone was growing. Also, epithelium and lamina propria tissues were covering the socket, but they were not continuous.

However, the alveolar socket of ovariectomized rats with pamidronate treatment for 7 days was filled with many inflammatory cells. At the periphery of the socket, remaining periodontal tissues were found without cell proliferation (Fig. 4)
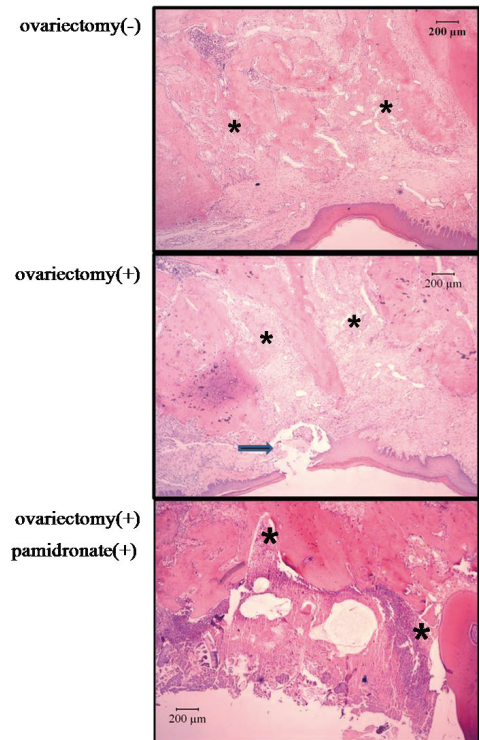
Figure 4. Alveolar socket at day 7 after tooth extraction. The alveolar socket without ovariectomy and pamidronate treatment is filled with growing immature woven bone (asterisks) and connective tissues without inflammatory cells apparently. The alveolar socket of ovariectomized rats without pamidronate treatment is filled with connective tissues at the central region and woven bone at the periphery (asterisks). Surface epithelial tissues are not continuous (arrow). Most portion of the alveolar socket of ovariectomized rats with pamidronate treatment is filled with neutrophilic inflammatory cells at the central region. At the periphery of the socket, remaining periodontal tissues (asterisks) are found without any proliferation. H-E stain

To localize VEGF expression in the alveolar socket at day 3 after tooth extraction, immunofluorescence staining was performed. Weak immunoreactivity against VEGF was observed in the remaining periodontal tissues and vessels under vasculogenesis in rats without any treatment. The immunoreactivity in ovariectomized rats was also found weak in tissues under regeneration as well as remaining periodontal tissues. The reactivity in ovariectomized rats with pamidronate treatment was strong in neutrophilic inflammatory cells (Fig. 5).
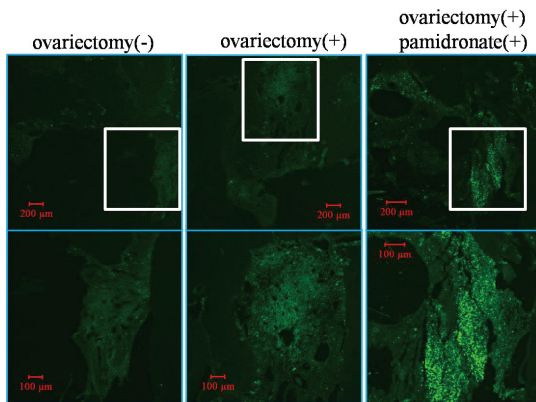
Figure 5. VEGF in the alveolar socket was localized at day 3 after tooth extraction using immunofluorescence staining. The reactivity in the socket without any treatment is weak. The rectangular area was magnified to show in the remaining PDL tissues. Weak reactivity is found in regenerative tissues of ovariectomized rats without pamidronate treatment. The reactivity in ovariectomized rats with pamidronate treatment is strong in inflammatory cells.

Discussion

For the *in vivo* study, standard osteoporosis animal model was made by bilateral surgical ovariectomy according to the Li et al. report[20], since bisphosphonatesare widely used for the prevention and treatment of osteoporosis in postmenopausal women. In the present study, 3.5 mg/kg/wk pamidronate was administered for 8 weeks. This was relatively long term therapy of high concentration and comparable to the adminstration of 3 mg/kg/d for 21 days for ONJ model in rats[21].

In the present *in vitro* study, not low but high concentration ($\geq 3.8 \times 10^{-3}$ M) of pamidronate treatment for 3 days also gave rise to reduced proliferation of periodontal ligament cells which are important in the regeneration of the alveolar socket after tooth extraction, suggesting a possibility that ONJ is implicated with high dose of bisphosphonate treatment. Also, this result was consistent with a report that exposure of high concentration ($\geq 6 \times 10^{-5}$ M) of pamidronate for 3 days resulted in the reduced human primary osteoblasts proliferation by apoptotic cell death[22]. Also, individuals receiving IV bisphosphonates are at significantly greater risk of ONJ than those using oral bisphosphonates. The reason for this greater risk has been speculated that it is because oral doses (1%) are not absorbed as easily as IV doses (50%). At least 5% of people receiving IV bisphosphonates develop ONJ[1,7]. Of all people diagnosed with ONJ, 94% were taking a high dose of IV bisphosphonate therapy, mostly pamidronate or zoledronic acid[8,23].

Many *in vitro* studies have shown that bisphosphonates inhibited angiogenesis through endothelial cells. Bisphosphonates inhibited integrin-mediated adhesion and migration[24,25], reduced proliferation, induced apoptosis and decreased capillary-like tube formation *in vitro* of endothelial cells (Fournier et al., 2002). Also, *in vivo*, Fournier et al[26]. found a 50% reduction in revascularization in rat prostate glands

after treated with zoledronate. However, many questions remain as to what are underlying mechanisms for the antiangiogenesis of bisphosphonates and whether the antiangiogenic properties play a major role in the pathogenesis of ONJ. In this study, it was hypothesized that bisphosphonates treatment may downregulate the VEGF expression during the healing process of the alveolar socket, leading to the reduction of vasculogenesis and healing ability, which subsequently can lead to delayed socket healing and increased time for potential bacterial infection. Surprisingly however, VEGF expression was increased by the treatment.

In *in vitro* studies, VEGF expression was examined after the pamidronate treatment. The level of VEGF expression was not changed at the low concentration of pamidronate (less than $\geq 3.8 \times 10^{-4}$ M), but the level increased at higher concentration ($\geq 3.8 \times 10^{-3}$ M). This was also confirmed at the translation level using Western blot. To further analyze changes in each VEGF isoform, mRNA levels of VEGF-A, -B and -C were determined. The treatment at high concentration (10 μg/ml) increased the VEGF-A and -B expression, whereas it little affected the VEGF-C expression. In *in vivo* study, Western blot result revealed that VEGF protein level was apparently increased by the pamidronate treatment for 3 days after ovariectomy and also confirmed by RT-PCR. The VEGF-A and -B mRNA level was increased by the pamidronate treatment for 3 and 7 days after ovariectomy, respectively. However, VEGF-C mRNA level was little affected. This *in vivo* and *in vitro* results suggest that VEGF is involved in socket healing somehow.

VEGF-A is the most potent and pivotal mediator

of angiogenesis and vasculogenesis and a highly specific mitogen for vascular endothelial cells. Conventional VEGF-A_{xxx} is angiogenic, while VEFG-A_{xxx}b isoform family is anti-angiogenic[27]. VEGF-A_{xxx}b isoforms resulting from this C′ terminal exon 8 splicing event, generate proteins of the same length as VEGF-A_{xxx} isoforms; however, the terminal six amino acids are different, since exon 8a codes for Cys-Asp-Lys-Pro-Arg-Arg and exon 8b for Ser-Leu-Thr-Arg-Lys-Asp. A balance of proangiogenic and antiangiogenic VEGF-A is necessary for normal development and function. Unregulated expression of VEGF-A_{165} is detrimental in tissue development. VEGF-A_{165}b play a significant role in the modulation of VEGF-A_{165}-driven responses[28,29]. In the present study, the mRNA expression level of VEGF$_{165}$b as well as VEGF$_{165}$ was increased by the pamidronate treatment. This suggested that proangiogenic activity was negatively regulated, which is detrimental in the alveolar socket healing. VEGF-B can induce inflammatory cell chemotaxis and has a role to play in inflammatory angiogenesis[16]. VEGF-C is mainly restricted to the lymphatic endothelium and has been implicated in the development of lymphatic vessels[17,18].

In the present study, the alveolar socket was undergoing active bone formation in the rats without ovariectomy and pamidronate treatment. The socket in the ovariectomized rats was also undergoing bone formation, but the maturity was less. This findings implied that ovariectomy negatively affected the socket healing. In one case with pamidronate treatment in ovariectomized rats, the socket was infected and infiltrated with numerous neutrophilic leukocytes. This finding was coincident with the report that bacterial infection at tooth extraction sites lead

to a delay in the wound healing process[30]. The immunofluorescence investigation revealed that reactivity against VEGF was weak in the ovariectomized rats without pamidronate treatment. However, strong reactivity was observed in the infiltrated leukocytes in ovariectomized rats with pamidronate treatment. In fact, neutrophils are able to produce different angiogenic molecules[31,32]. and play a role as cellular effectors of pathologic angiogenesis[33]. In addition, infection has been suggested as a cause of ONJ. ONJ shows heavy infiltration of inflammatory cells and bacterial flora[34]. The oral cavity has unique environmental features which are vulnerable to ONJ. The intimate relationship of teeth to the jaws allows a portal of entry for microbes and other inflammatory products to the underlying bone, a situation that is not found in any other part of the body[35]. Bisphosphonates promote jaw osteonecrosis through facilitating bacterial colonization. Actinomyces colonization of exposed bone in the bisphosphonate treated group was increased statistically significantly (11/18; 61%) when compared to the nontreated group (2/11; 18%, P = 0.021)[36]. Ongoing periodontal disease was present in a significant number of ONJ patients[37].

It is not likely that the antiangiogenic property of bisphosphonates is the sole cause for the development of ONJ[38]. In the present study, RT-PCR, Western blot and immunofluorescent study revealed that VEGF was increased by the pamidronate treatment. These findings were consistent with the report that VEGF participates in some pathological courses such as thrombosis, inflammation and ischemia[39]. Particularly, this study revealed that VEFG-B was increased by the pamidronate treatment. When considering its roles of inflammatory cell chemotaxis and inflam-

matory angiogenesis[16], the increased expression can be related with inflammatory reaction in the alveolar socket, which can be further implicated in underlying mechanism of ONJ. Inflammatory cells have been shown in bone biopsies from ONJ patients. T cell activation and proliferation in ONJ was suggested as a hypothesis[35]. Nitrogen-containing bisphosphonates such as pamidronate are well-known to cause a flu-like acute phase reaction in many patients, which has been linked to the intracellular accumulation and subsequent presentation to T cells of isopentenyl pyrophosphate products accrued from the inhibition of the enzyme farnesyl pyrophosphate (FPP)-synthase by the N-containing bisphosphonates[40]. Reduced viability, growth, and migration of cells in the bone and soft tissues were causative for ONJ. Exposed jawbone after tooth extraction can be a reliable cofactor[40].

All together, this study suggested that uncontrolled bisphosphonate treatment may induce ONJ by altering VEGF regulation as another mechanism of ONJ.

References

1. American Dental Association Council on Scientific Affairs. Dental management of patients receiving oral bisphosphonate therapy. J Am Dent Assoc, 137:1144–1150, 2006. https://doi.org/10.14219/jada.archive.2006.0355.

2. Drake M, Clarke BL, Khosla S: Bisphosphonates: Mechanism of action and role in clinical practice. Mayo Clin Proc, 83:1032–1045, 2011.https://doi.org/10.4065/83.9.1032

3. George EL, Lin YL, Saunders MM: Bisphosphonate-related osteonecrosis of the jaw: a mechanobiology perspective. Bone Rep, 15;8:104-109, 2018. doi:

10.1016/j.bonr.2018.03.003.

4. Heron P, Abellán-Flos M, Salmon L, Sygusch J: Bisphosphonate inhibitors of mammalian glycolytic aldolase. J Med Chem, 2018, doi: 10.1021/acs.jmedchem.8b01000. [Epub ahead of print]

5. Cartsos VM, Zhu S, Zavras AI: Bisphosphonate use and the risk of adverse jaw outcomes: A medical claims study of 714,217 people. J Am Dent Assoc, 139:23−30, 2008. https://doi.org/10.14219/jada.archive.2008.0016.

6. Sedghizadeh PP, Stanley K, Caligiuri M, Hofkes S, Lowry B, Shuler CF: Oral bisphosphonate use and the prevalence of osteonecrosis of the jaw. J Am Dent Assoc, 140:61-66, 2009. https://doi.org/10.14219/jada.archive.2009.0019.

7. American Dental Association. Osteonecrosis of the jaw [on-line]. Available at http://www.ada.org/2995.aspx?currentTab=1 (Dentist version) 2008.

8. Clarke B, Koka S: Bisphosphonate-associated osteonecrosis of the jaw. American College of Rheumatology Hotline [on-line]. Available at http://www.rheumatology.org/publications/hotline/0606onj.asp 2006.

9. Ruggiero SL, Fantasia J, Carlson E: Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 102:433-441, 2006. DOI: 10.1016/j.tripleo.2006.06.004

10. Reid IR, Bolland MJ, Grey AB: Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity ? Bone, 41:318-320, 2007. DOI: 10.1016/j.bone.2007.04.196.

11. Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, 86:353−364, 1996. DOI:https://doi.org/10.1016/S0092-8674(00)80108-7.

12. Carmeliet P: Angiogenesis in health and disease. Nat Med, 9:653-660, 2003. DOI: 10.1038/nm0603-653.

13. Lyer S, Acharya KR: Tying the knot: The cystine signature and molecular-recognition processes of the vascular endothelial growth factor family of angiogenic cytokines. FEBS J, 278(22):4304-4322, 2011. https://doi.org/10.1111/j.1742-4658.2011.08350.x.

14. Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO: VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res, 64:7822−7835, 2004. DOI: 10.1158/0008-5472.CAN-04-0934.

15. Woolard J, Bevan HS, Harper SJ, Bates DO: Molecular diversity of VEGF-A as a regulator of its biological activity. Microcirculation, 16(7):572-592, 2009. DOI: 10.1080/10739680902997333.

16. Detoraki A, Staiano RI, Granata F, Giannattasio G, Prevete N, de Paulis A, Ribatti D, Genovese A, Triggiani M & Marone G: Vascular endothelial growth factors synthesized by human lung mast cells exert angiogenic effects. J Allergy Clin Immunol, 123:1142−1149, 2009. https://doi.org/10.1016/j.jaci.2009.01.044.

17. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K: Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92, 3566−3570, 1995. https://doi.org/10.1073/pnas.92.8.3566.

18. Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K & Wilting J: VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 188:96−109, 1997. DOI: 10.1006/dbio.1997.8639.

19. Ficarra G, Beninati F: Bisphosphonate-related osteonecrosis of the jaws: an update on clinical, pathological and management aspects. Head Neck Pathol, 132-140, 2007. doi: 10.1007/s12105-007-0033-2.

20. Li Y, Li X, Song G, Chen K, Yin G, Hu J: Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats. Clin Oral Implants Res, 23(9):1038-1044, 2012. doi: 10.1111/j.1600-0501.2011.02252.x.

21. López-Jornet P, Camacho-Alonso F, Molina-Miñano F,

Gómez-García F, Vicente-Ortega V: An experimental study of bisphosphonate-induced jaws osteonecrosis in Sprague-Dawley rats. J Oral Pathol Med, 39(9):697-702, 2010. doi: 10.1111/j.1600-0714.2010.00927.x.

22. Marolt D, Cozin M, Vunjak-Novakovic G, Cremers S, Landesberg R: Effects of pamidronate on human alveolar osteoblasts in vitro. J Oral Maxillofac Surg, 70(5):1081-1092, 2012. doi: 10.1016/j.joms.2011.05.002.

23. Russell RG: Bisphosphonates: mode of action and pharmacology. Pediatrics, 119 Suppl 2:S150-162. 2007. DOI: 10.1542/peds.2006-2023H

24. Wood, J. Bonjean K, Ruetz S, Bellahcène A, Devy L, Foidart JM, Castronovo V, Green JR: Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J Pharmacol Exp Ther, 302: 1055-1061, 2002. DOI: 10.1124/jpet.102.035295.

25. Bezzi M, Hasmim M, Bieler G, Dormond O, Rüegg C: Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. J Biol Chem, 278(44):43603-43614. DOI: 10.1074/jbc.M308114200.

26. Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, Clézardin P: Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res, 62: 6538 − 6544, 2009. PMID: 12438248.

27. Freitas-Andrade M, Carmeliet P, Stanimirovic DB, Moreno M: VEGFR-2-mediated increased proliferation and survival in response to oxygen and glucose deprivation in PlGF knockout astrocytes. J Neurochem, 107:756-767, 2008. doi: 10.1111/j.1471-4159.2008.05660.x.

28. Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE: Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest, 111:707 − 716, 2003. DOI: 10.1172/JCI17423.

29. Bevan HS, van den Akker NM, Qiu Y, Polman JA, Foster RR, Yem J, Nishikawa A, Satchell SC, Harper SJ, Gittenberger-de Groot AC, Bates DO: The alternatively spliced anti-angiogenic family of VEGF isoforms VEGFxxxb in human kidney development. Nephron Physiol, 110(4):57-67, 2008. doi: 10.1159/000177614.

30. Mawardi H, Giro G, Kajiya M, Ohta K, Almazrooa S, Alshwaimi E, Woo SB, Nishimura I, Kawai T: A role of oral bacteria in bisphosphonate-induced osteonecrosis of the jaw. J Dent Res, 90(11):1339-1345. 2011. doi: 10.1177/0022034511420430.

31. Gong Y, Koh DR: Neutrophils promote inflammatory angiogenesis via release of proformed VEGF in an in vivo corneal model. Cell Tissue Res, 339:437-448, 2010. doi: 10.1007/s00441-009-0908-5.

32. Walczak C, Gaignier F, Gilet A, Zou F, Thornton SN, Ropars A: Aldosterone increases VEGF-A production in human neutrophils through PI3K, ERK1/2 and p38 pathways. Biochim Biophys Acta, 1813(12):2125-2132, 2011. doi: 10.1016/j.bbamcr.2011.07.010.

33. Gaudry M, Brégerie O, Andrieu V, El Benna J, Pocidalo MA, Hakim J: Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood, 15;90(10):4153-4161,1997. PMID: 9354686.

34. Han JW: Bisphsophonate related osteonecrosis of the jaws: report of two cases. Imaging Sci Dent, 41(3):129-134, 2011. DOI: 10.5624/isd.2011.41.3.129.

35. Landesberg R, Woo V, Cremers S, Cozin M, Marolt D, Vunjak-Novakovic G, Kousteni S, Raghavan S: A role of oral bacteria in bisphosphonate-induced osteonecrosis of the jaw. Potential pathophysiological mechanisms in osteonecrosis of the jaw. Ann N Y Acad Sci, 1218:62-79, doi: 10.1111/j.1749-6632.2010.05835.x. 2011.

36. Kos M, Brusco DJ, Kuebler W, Engelke W: Clinical comparison of patients with osteonecrosis of the jaws, with and without a history of bisphosphonates administration. Int J Oral Maxillofac Surg, 39: 1097 − 1102, 2010. doi: 10.1016/j.ijom.2010.04.054.

37. 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: 1567-1575, 2005. DOI: 10.1016/j.joms.2005.07.010.

38. Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B: American Association of Oral and Maxillofacial Surgeons. Position paper on bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg, 67(Suppl. 5): 2−12, 2009. doi: 10.1016/j.joms.2009.01.009.

39. Xiong Y, Han XF, Luo JC: non-angiogenic functions of vascular endothelial growth factors. Sheng Li Ke Xue Jin Zhan 42:6-10, 2011. PMID: 21595180.

40. Roelofs AJ, Thompson K, Gordon S, Rogers MJ: Molecular mechanisms of action of bisphospho-nates: current status. Clin Cancer Res, 12(20Pt 2): 6222s−6230s. 2006. DOI: 10.1158/1078-0432.CCR-06-0843.

41. Holtmann H, Lommen J, Kübler NR, Sproll C, Rana M, Karschuck P, Depprich R: Pathogenesis of medication-related osteonecrosis of the jaw: a comparative study of in vivo and in vitro tri-als. J Int Med Res, 46(10):4277-4296. 2018. doi: 10.1177/0300060518788987.

한글초록

골다공증 유발 백서에서 Bisphosphonate에 의한 발치창내 VEGF 발현 변화

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비스포스포네이트는 파골세포의 활성을 억제하여 골 손실을 억제하며 골다공증 치료에 널리 이용되고 있다. 비스포스포네이트는 특히 악골에서 골괴사를 일으키는 심각한 부작용을 가지나 그 부작용에 대한 원인 및 기전은 뚜렷이 밝혀져 있지 않다. 발치와 치유에는 혈관 성장이 필수적이다. 따라서 본 연구는 비스포스포네이트가 혈관성장인자의 발현을 억제함으로써 발치와 치유에 장애를 초래하며 궁극적으로 골괴사 요인이 됨을 가정하고 다음과 같은 실험 결과를 얻었다.

골다공증을 유발하기 위하여 백서 암컷에서 외과적으로 난소를 제거하고 16주 후 대조군으로 이용하였다. 실험군은 난소 절제 후 8주에 격일로 pamidronate(3.5 mg/Kg/wk)를 투여하였다. 16주에 상악제1대구치를 발치 후 3일, 7일에 희생하였다. 치주인대세포는 발치와 재생에 중요하므로 생쥐 치주인대세포를 배양하여 pamidronate를 처리하였다. *in vitro* 실험에서 치주인대세포는 고농도(10 μg/ml) 처리에서 VEGF-A, -B가 모두 증가되었으나 VEGF-C는 변화가 없었다. *in vivo* 실험에서 VEGF 발현은 *in vitro*와 유사하였다. 비스포스포네이트 투여군의 발치창에서는 중성백혈구의 침윤이 뚜렷하였으며 백혈구는 VEGF에 대한 강한 면역반응을 나타내었다. 이상의 결과는 VEGF-A 또는 -B가 염증세포를 유인하여 골괴사를 유발하는 인자로 작용할 수 있음을 시사하였다.

주제어: VEGF, 비스포스포네이트, 골괴사