Differential impairment of vascularization and angiogenesis in bisphosphonate-associated osteonecrosis of the jaw-related mucoperiosteal tissue

Falk Wehrhan, MD, DMD, a Phillip Stockmann, MD, DMD, a Emeka Nkenke, MD, DMD, PhD, a Karl A. Schlegel, MD, DMD, PhD, a Arndt Guentsch, DMD, b Theresia Wehrhan, a Friedrich W. Neukam, MD, DMD, PhD, a and Kerstin Amann, MD, c Erlangen and Jena, Germany

UNIVERSITY OF ERLANGEN-NUREMBERG AND UNIVERSITY OF JENA

Objectives. Impaired vascularization in the etiopathology of aminobisphosphonate-associated osteonecrosis of the jaw (BONJ) is assumed, but evidence is lacking. This immunohistochemical study differentiated vascularization and angiogenesis in BONJ-adjacent mucoperiosteal tissue.

Study design. Twenty BONJ (after zoledronate treatment) and 20 control mucoperiosteal tissue samples were processed with an autostaining-based alkaline phosphatase-antialkaline phosphatase staining kit. Vascularization was assessed by CD31 staining and angiogenesis-related neovessels by CD105 staining. The ratio of stained capillary area to total area of visible field was assessed. Statistics included Bonferroni adjustment.

Results. CD31-stained microvessels were detected in each section and CD105-stained neovessels in each control. BONJ-adjacent mucoperiosteal tissue showed significantly fewer CD105-positive vessels in capillary areas ($P < .05$) than control samples. CD31-stained capillary area was not significantly reduced in mucoperiosteal BONJ-samples.

Conclusions. Angiogenesis is impaired in BONJ-related mucoperiosteal tissue, but vascularization remains unaffected. Vessel remodeling and neovessel formation is delayed in BONJ, resulting in impaired tissue regeneration of bisphosphonate-exposed oral mucosa. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:216-221)

An understanding of the underlying pathophysiology of aminobisphosphonate (BP)-associated osteonecrosis of the jaw (BONJ) is limited.1,2 Numerous attempts have been made to explain why BONJ is restricted to the jaws, but an accepted model of the pathology is still lacking.3,4 Early descriptions of BONJ as an avascular necrosis lacked evidence of reduced vascularization or diminished blood supply in the affected jaw tissue.2 Those descriptions have been partly revised because of later demonstrations of patent vascularization in BONJ.3 Histomorphometric analyses showed increased trabecular thickness but did not demonstrate reduced vascularization in BONJ-related jaw bones.6 Indeed, BP treatment was found to improve vascularization of bone in cases of femur head necrosis, and BP was described as accelerating fracture repair in extracranial skeletal bone.7,9 However, several studies showed dose-dependent cytotoxicity of BP of endothelial cells in vitro.10 Reduced endothelial cell proliferation and impaired endothelial cell migration during angiogenesis have been described in vitro after administration of zoledronic acid.10-12 Oral mucosal healing after BP treatment was shown to be impaired.13,14 One clinical study reported increased vascular endothelial growth factor (VEGF) expression in BONJ-affected oral mucosa despite reduced VEGF expression in BP-related oral mucosa in the absence of BONJ.15 Therefore,
CD31 is widely accepted as a marker of vascularization, including microvessels.\textsuperscript{16-18} Quantitative descriptions of vascularization in several tissues by estimating CD31-associated capillary area by means of histomorphometry are common.\textsuperscript{18,19} Histomorphometric analysis of CD31-stained capillaries in BONJ-affected mucosa could, therefore, help to determine if vascularization in BONJ-related tissue is impaired. Impairment of angiogenesis has been implicated in BONJ development. Therefore, information differentiating vascularization and neovascularization (angiogenesis) in BONJ-related oral mucosa is of interest. CD105, a surface structure motif of the betaglycan receptor transforming growth factor \( \beta \) receptor III (TGF\( \beta \)-RIII), is expressed predominantly in endothelial cells.\textsuperscript{20} Tumor-associated neovessels and wound healing-related newly formed capillaries have been shown to specifically express CD105.\textsuperscript{19,21,22} CD105 is strictly associated with newly formed blood vessel endothelial cells.\textsuperscript{22,23} During skin healing, angiogenesis-related CD105 was expressed 14-18 days after the beginning of capillary outgrowth from preexisting vessels.\textsuperscript{19,22}

The aim of the present study was to differentially assess vascularization and angiogenesis in BONJ-affected mucoperiosteum compared with noncompromised oral mucoperiosteum. For this purpose, CD31\textsuperscript{*} and CD105-stained capillaries were quantitatively assessed by performing histomorphometry of tissue samples of BONJ-affected and normal mucoperiosteum.

**MATERIALS AND METHODS**

**Patients and material harvesting**

This study included oral mucoperiosteal specimens from 40 patients. Of these, 20 specimens were from periodontal soft tissue adjacent to clinically and histologically confirmed BONJ of 20 consecutively treated patients undergoing radical sequestrectomy. Tissue specimens were taken as part of the tissue samples provided for routine histopathologic diagnostics. The necrotic tissue itself was excluded from the analysis. This study was approved by the local Ethical Committee of the University of Erlangen-Nuremberg (ref. no. 4,272). Criteria for specimen inclusion were intravenous administration of zoledronate or pamidronate for \( \geq 12 \) months and clinical evidence of an exposed jaw bone for \( \geq 8 \) weeks. Specimens from patients previously treated with radiotherapy were excluded. Details of the patients, surgical treatment, and follow-up period were previously documented in a clinical study by our group.\textsuperscript{24} Control samples included 20 alveolar mucoperiosteal specimens harvested during intraoral den-toalveolar surgery in patients with no history of BP treatment and who presented with no clinical signs of intraoral inflammatory processes. Each specimen measured on average \( 5 \times 3 \times 3 \) mm, and was immediately flash frozen at -80°C in liquid nitrogen. Thereafter, specimens were fixed in 4% paraformaldehyde, incubated in graded alcohol solutions, and embedded in paraffin.

**Immunohistochemical staining**

Paraffin-embedded tissue samples were processed for immunohistochemistry.\textsuperscript{25} Antibodies and dilutions were used as follows: CD31: polyclonal mouse IgG antihuman CD31 antibody (anti-CD31, MCA 1812; Serotec, Düsseldorf, Germany) at a dilution of 1:100; CD105: polyclonal rabbit IgG antihuman CD105 antibody (anti-CD 105, Ab-3 [SN6h]; NeoMarkers, Fremont, CA, USA; dilution of 1:100). Secondary antibody was used according to the staining kit (biotinylated polyclonal goat antirabbit IgG (E 0466; DakoCytomation, Hamburg, Germany; dilution 1:100). Visualization was performed using Fast Red solution and localization by biotin-associated activation of the staining kit (alkaline phosphatase-antialkaline phosphatase method; ChemMate-Kit, Dako), followed by incubation in hematoxylin for nuclear counterstaining. Two tissue samples per group were processed per immunohistochemical staining, one for experimental staining and the other as a negative control (replacement of primary antibody with nonspecific isotype IgG of the primary antibody). Consecutive sections were processed for CD31 and CD105 staining. Serial sections were used exclusively in this study. A known positive-staining sample was also included in each series as a positive control. The serial positive-staining sections were taken from a specimen of oral mucoperiosteum exhibiting both CD31 and CD105 staining. The patient’s history was negative for any BP treatment and for any intraoral inflammatory processes.

**Semiquantitative immunohistochemical analysis**

Sections were examined qualitatively under a bright-field microscope (Axioskop; Zeiss, Jena, Germany) at \( \times 200 \) magnification for localization of stained capillaries. In healthy mucoperiosteal control samples, subepithelial tissue was observed, including connective, submucosal, and periosteal structures. Mature bone tissue, including osteocytes, was excluded from any analysis. In BONJ-related tissue samples, soft tissue adjacent to the necrotic zone was identified, and 3 visual fields per section for each sample were digitized.
at ×200 magnification using a CCD camera (AxioCam 5; Zeiss) and the AxioVision program (Zeiss). For this purpose, randomized systematic subsampling was performed as previously described.\textsuperscript{25} Semiquantitative histomorphometric analysis of vascularization was performed using established methods (NIH Image V1.61; U.S. National Institutes of Health; and Scion Image PC; Scion Corporation, Frederick, MD, USA) at ×200 magnification. The targeted parameters were the capillary-marked areas per visual field. Differential vascularization- and angiogenesis-related capillary staining was performed for CD31- and CD105-positive capillaries on consecutive immunohistochemistry sections.

\textbf{Statistical analyses}

Single measurements per individual and study group were aggregated before analysis. Descriptive statistical analyses of relative capillary area data were performed using the arithmetic mean and standard deviation (SD). Graphical descriptions were created using boxplots representing the median and interquartile range. Confirmatory comparisons between treatment and control groups were made using generalized estimating equations (GEE) with “study group” and “subject identification” as independent factors for appropriate analysis of repeated measurements per individual. Multiple \( P \) values were adjusted according to Bonferroni by multiplying...
each obtained $P$ value by the number of performed confirmatory tests ($n = 10$). Two-sided, adjusted $P$ values of $P \leq .05$ were considered to be significant. All calculations were made using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Capillaries were seen in BONJ-related mucoperiosteal specimens and healthy jaw connective tissue. In normal jaw mucoperiosteal tissue and in BONJ-related samples, vascularization was clearly detectable by CD31-positive capillary staining in both groups (Figs. 1, A, and 2, A). No morphologic differences regarding the capillaries were seen between BONJ-affected and nonBP-exposed mucoperiosteal tissue. Vascular endothelial cell layers in both groups showed similar shapes and thicknesses. Newly formed angiogenesis-related capillaries, which stained positively for CD105, were detected in the nonBP-exposed mucoperiosteal tissue and to a lesser extent in BONJ-related samples (Figs. 1, B, and 2, B). The mucoperiosteal soft tissue exhibited variable numbers of cell layers, including inflammatory infiltrates within the connective tissue layers.

The relative capillary area of CD31-associated vascularization was slightly reduced in BONJ-related samples compared with normal mucoperiosteal tissue (Fig. 3). However, these differences in vascularization were not significant (Fig. 3; Table I). CD105-positive relative capillary area was significantly diminished in the BONJ-related tissue samples compared with nonBP-exposed mucoperiosteal tissue ($P < .05$; Fig. 3; Table I).

Because consecutive histologic sections were used to analyze CD105 and CD31 staining, vascularization and angiogenesis data are directly correlated. Whereas in normal mucoperiosteal tissue angiogenesis-related capillary area represented 50% of the total capillary area, in the BONJ-group angiogenesis represented only 25% of the total capillary area (Table I).

DISCUSSION

These results show reduced angiogenesis in BONJ-related oral mucoperiosteal tissue compared with normal tissue, as shown by the significantly ($P < .05$) decreased CD105-positive relative capillary area. The inhibition of angiogenesis without significant effect on vascularization in BONJ was demonstrated for the first time in this study.

Reduced angiogenesis is consistent with the clinical finding of local dentoalveolar trauma-related incidence in BONJ and prolonged mucosal healing.²⁶,²⁷ Whereas mature mucosa in BP-treated patients seems to be sufficiently vascularized, reduced angiogenesis causes delay and impairment of the remodeling process when neovascularization is required.²⁷ Other reports describe decreased expression of angiogenesis-inducing VEGF in the oral mucosa of patients treated with BP in the absence of ONJ, despite a massive increase of VEGF in BONJ mucosal lesions.¹⁵ VEGF and angiogenesis are known to be stimulated by tissue hypoxia and inflammation. Therefore, the interpretation of increased VEGF expression and increased expression of vessel-related proteins in BONJ-mucosal lesions is difficult and seems to involve the reparative capacity and angiogenesis of the BP-exposed tissue.²⁸

Table I. Numeric calculations of the relative capillary area (%) in BONJ and control mucoperiosteal tissue, differentiating CD31- and CD105-staining capillaries

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<th>BONJ (median)</th>
<th>Control (median)</th>
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<tr>
<td>CD31</td>
<td>9.5 (7.2)</td>
<td>11.3 (5.1)</td>
</tr>
<tr>
<td>CD105</td>
<td>2.4 (1.6)</td>
<td>6.1 (5.2)</td>
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Values are median (interquartile range) BONJ-related CD105-positive capillary area is significantly ($P < .05$) reduced compared with the control mucoperiosteal tissue.

Fig. 3. Quantitative estimate of the relative capillary area positively staining for either CD31 or CD105. The median and the interquartile ranges, as well as the maximum and minimum of each category are shown as boxplots. Angiogenesis-related CD105-positive capillary area in BONJ-affected mucosa was significantly reduced compared with control samples ($P < .05$).
These observations suggest that angiogenesis is specifically impaired in the absence of secondary inflammation and is an underlying mechanism of impaired vessel remodeling. This hypothesis is substantiated by the specific impairment of the migration and proliferation of vascular endothelial cells in vitro and reduced angiogenesis during early healing of extraction sockets in mice after BP administration.\textsuperscript{10-12,27} These findings would explain why angiogenesis is specifically reduced but vascularization is not impaired.

The results of the present study demonstrating no significantly reduced vascularization in BONJ-adjacent mucoperiosteal tissue compared with nonBP-exposed oral mucosa are consistent with recent results reported by other groups describing no reduction of vascularization in BONJ-related jaw.\textsuperscript{29} An experimental study in dogs showed patent vascularization in BP-compromised jaw matrix tissue.\textsuperscript{30} Furthermore, no histomorphologic changes in vessels within BP-exposed tissue have been described.\textsuperscript{5,31} Indeed, in extracranial tissues BP was even reported to increase vascularization and to accelerate fracture in some studies.\textsuperscript{7-9,32}

The underlying mechanism of impaired angiogenesis could involve the unique biologic features of jaw tissue, because BONJ has been described exclusively in jaws. Receptor activator of nuclear KB (ligand) [RANK(L)], which is critically involved in angiogenesis, is differentially expressed in jaw and extracranial vascular and osteoblast progenitors.\textsuperscript{33,36} BP treatment can suppress RANK(L) expression.\textsuperscript{37-40} Recently, RANK(L) was shown to be suppressed in mucoperiosteal tissue of ONJ-adjacent sites at the protein and mRNA levels.\textsuperscript{41} Because endogenous RANK(L) expression is known to be higher in craniofacial pluripotent progenitor cells compared with extracranial progenitors, BP-related suppression of RANK(L)-dependent cellular signaling is likely to affect craniofacial vascular progenitor cell differentiation more than extracranial cell signaling.\textsuperscript{34} RANK(L) suppression-related impairment of angiogenesis is consistent with the findings of our study of reduced TGFβR-III-associated neovessel formation, whereas mature vascularization is not affected. The suggestion of specific impairment of angiogenesis should be clarified in the future, by analyzing cellular mechanisms of vascular differentiation in oral mucoperiosteal tissue during BP exposure.

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REFERENCES

Reprint requests:
Falk Wehrhan, MD, DMD
Department of Oral and Maxillofacial Surgery
Friedrich-Alexander-University of Erlangen
Glueckstrasse 11
91054 Erlangen
Germany
falk.wehrhan@uk-erlangen.de