Immunohistochemial evaluation of sarcoglycans and integrins in gingival epithelium of multiple myeloma patients with bisphosphonate-induced osteonecrosis of the jaw

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Abstract. Osteonecrosis of the jaw (ONJ) is an adverse outcome associated to bisphosphonate treatment. However, it is not known whether the ONJ lesion originates in the bone, or whether it may initiate in the oral mucosa. The aim of our study was to evaluate the pattern of basal lamina of oral mucosa after bisphosphonate administration and to analyze the structural damage of the mucosa in ONJ patients, and in subjects treated with bisphosphonates without osteonecrosis. By immunohistochemistry, we evaluated changes in basement membrane by expression of signalling proteins, laminin, and type IV collagen. All tested proteins were almost absent in basal lamina and mucosa of subjects treated with bisphosphonates without osteonecrosis, whereas in mucosa of patients with ONJ, they showed a clearly detectable pattern of the same proteins, specifically in basal lamina, but less in comparison to control samples. Moreover, in pathological mucosa, the clearly detectable staining pattern for VEGF indicated a massive neoangiogenesis. Bisphosphonates induce changes in expression of proteins also in oral mucosa. The increase of these proteins in basal lamina, and the neo-angiogenesis, concomitant with formation of the lesion, could indicate a compensative behaviour in the remodelling of the gingival mucosa in order to restore the epithelial architecture.

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Introduction

Bisphosphonates (BPs) are the current standard care for preventing skeletal related events. Since 2003, numerous reports of bisphosphonate-induced osteonecrosis of the Jaws (ONJ) have appeared in literature (1). Several theories have been proposed regarding the pathoetiology of ONJ, e.g. that necrosis is related to an over-suppression of bone turnover by BPs (2) or that BPs decrease angiogenesis (3,4).

Different definitions for ONJ have been proposed and all include exposure of maxillary or mandibular bone, but a breech in the oral mucosa is an absolute requirement. At the present time it is in fact not definitively known whether the ONJ lesion originates in the bone, or whether it may initiate in the mucosa (5).

However, toxicity of BPs to gastric and intestinal mucosal cells has been documented, and the mechanism of damage was independent of microvascular injury, possibly due to disruption of surface active phospholipids within the mucosal layer (6). Twiss *et al* (7) observed that, in presence of calcium, pamidronate forms insoluble complexes that deposit on the cell surface, exposing gastric mucosa to locally increased and cytotoxic concentrations of the drug (7,8); while Suri *et al* (9) postulated that the toxic effects on intestinal cells are related to disruption of the mevalonate pathway, which reduces production of cholesterol precursors and interferes with cell membrane biosynthesis.

Although several studies have been carried out on these drugs data are almost absent on structural and proteic modifications that they cause in the oral mucosa. On these basis, we evaluated changes in localization and expression of proteins which play a key role in the maintenance of cellular viability and signalling i.e. vinculin-talin-integrin system and sarcoglycan sub-complex, and in the integrity of basement membrane i.e. laminin, and type IV collagen. Moreover, in order to verify the behaviour of the vascular component, VEGF was also tested.

Basement membranes are thin, specialized extracellular matrices that are important for providing mechanical stability

to tissue and also provide signals that influence cellular polarity and guide cell migration (10). The extracellular matrix molecule laminin is a large heterodimeric glycoprotein and it is required for basement membrane architecture, providing mechanical support to adjacent cells (11,12).

The vinculin-talin-integrin system constitutes a well known protein machinery which in skeletal muscle fibers forms the costameres (13-17). Vinculin head is known to bind to α-actin and talin, whereas vinculin tail is known to bind to paxillin, F-actin and phosphatidylinositol 4,5 bisphosphate (18).

Integrins are transmembrane α/ß heterodimers that can bind to many extracellular matrix molecules. Integrins are important for cell adhesion and migration, and can have roles in matrix assembly and remodelling (19). The vinculin-talin-integrin system is present in numerous tissue and it has the characteristics of atypical focal contacts, binding only intermediate filaments and not thin filaments (20-24), and integrins are its transmembrane part.

The α7B and ß1D are the most common integrins found in adult skeletal and smooth muscle (25,26); however, we tested these isoforms in gingival mucosa in order to study their behaviour in vascular structures during ONJ. All integrin isoforms are involved in a myriad of biological processes such as haemostasis, immune response, wound healing, angiogenesis, embryogenesis, and tumorigenesis (22,27).

Similarly to the vinculin-talin-integrin system, the sarcoglycan sub-complex (SGC) represents a system that plays an important role in cell-cell binding and in cell viability in many cellular types (28). In particular, the SGC, composed of six proteins (α-, ß-, γ-, δ-, ε-, and ζ-sarcoglycan) stabilizes the cell membrane during cellular activity (29,30). It was demonstrated that α- and γ-sarcoglycan are expressed exclusively in skeletal and cardiac muscle, whereas ß- and δ-sarcoglycans are more widely expressed (31).

Type IV collagen polymers is though to be key structural element of the basement membrane and is usually accompanied by other glycoproteins.

Our aim was to characterize the distribution and expression of different mucosal proteins after bisphosphonate administration and to evaluate the structural damage of the mucosa in ONJ patients, using immunohistochemical techniques.

Materials and methods

Patients. Samples of human gingival epithelium were obtained from 10 patients treated with bisphosphonate which did not show ONJ, and from 10 patients treated with bisphosphonates affected by ONJ. These samples were compared with oral mucosa of 10 control subjects who had undergone oral procedures for other reasons. The age of the patients ranged between 30 and 81 years and all gave their informed consent.

The procedures followed were in accordance with the principles outlined in the Helsinki Declaration of 1975.

Patients treated with bisphosphonates without ONJ (4 females and 6 males; median age 72±11 years) were affected by multiple myeloma (the paraprotein heavy chain was IgG in all patients; 5 patients were in stage II and 5 in stage III according to the Durie Salmon classification). They were treated with zoledronate (4 mg i.v. once a month). The duration from first use of the drug was 36±12.3 months. In this group biopsy was made the day before the last administration of bisphosphonates.

In ONJ patients last bisphosphonate administration was made 2-10 days before the biopsy was performed. The patients of this group were 5 females, 5 males; median age 70.1±12 years. Four patients were in stage II and 6 patients in stage III according to the Durie Salmon classification. The paraprotein heavy chain was IgG in all patients. All patients presented with an area of exposed bone and pain or swelling. The mandible and maxilla were the only bones involved in the exposure. Three cases of exposure occurred exclusively in the mandible, 3 exclusively in the maxilla and 4 simultaneously in the mandible and maxilla. The posterior mandible in the area of the molars was the most common site of exposure (n=3). All cases occurred after dental disease, oral surgical treatment or a traumatic injury of the oral mucosa by inadequate dentures. They received zoledronate (4 mg i.v. once every 3 weeks or once a month). The time from the first use of the drug to the first recognition of exposed bone was 33±15.1 months.

None of the studied patients had received chemotherapy at the time of biopsy. Patients with acute or chronic infections, diabetes, liver, kidney and inflammatory diseases were excluded from the study.

Specimens of oral mucosa were taken during surgical procedures, consisting of the removal of the sequestred bone, in patients affected by ONJ.

In the other groups, biopsies of crestal mucosa were performed during several oral surgical procedures (i.e. dental extractions, and implantology). No adrenaline was used for local anesthesia.

Immunohistochemistry. The specimens were analyzed using immunohistochemistry. Biopsies were fixed in 3% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, for 2 h at room temperature. They were then washed extensively with 0.2 M phosphate buffer, pH 7.4, and then with phosphate-buffered saline (PBS), containing 12 and 18% sucrose. The samples were snap-frozen in liquid nitrogen and 20 μm sections were prepared in a cryostat for their use in a protocol for immunofluorescence. The sections were placed on glass slides that were coated with 0.5% gelatin and 0.005% chromium potassium sulphate.

To block non-specific binding sites and to permeabilize the membranes, the sections were preincubated with 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS at room temperature for 15 min. Finally, the sections were incubated with primary antibodies.

The following antibodies for triple fluorescence were used: anti-α-sarcoglycan diluted 1:100, anti-ß-sarcoglycan diluted 1:200, anti-γ-sarcoglycan diluted 1:100, anti-δ-sarcoglycan diluted 1:50, anti-ε-sarcoglycan diluted 1:100 (all from Novocastra Laboratories, Newcastle upon Tyne, UK); anti-vinculin diluted 1:100, and anti-type IV collagen diluted 1:100 (both from Sigma Chemicals, St. Louis, MO); anti-VEGF diluted 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-α7B-integrin diluted

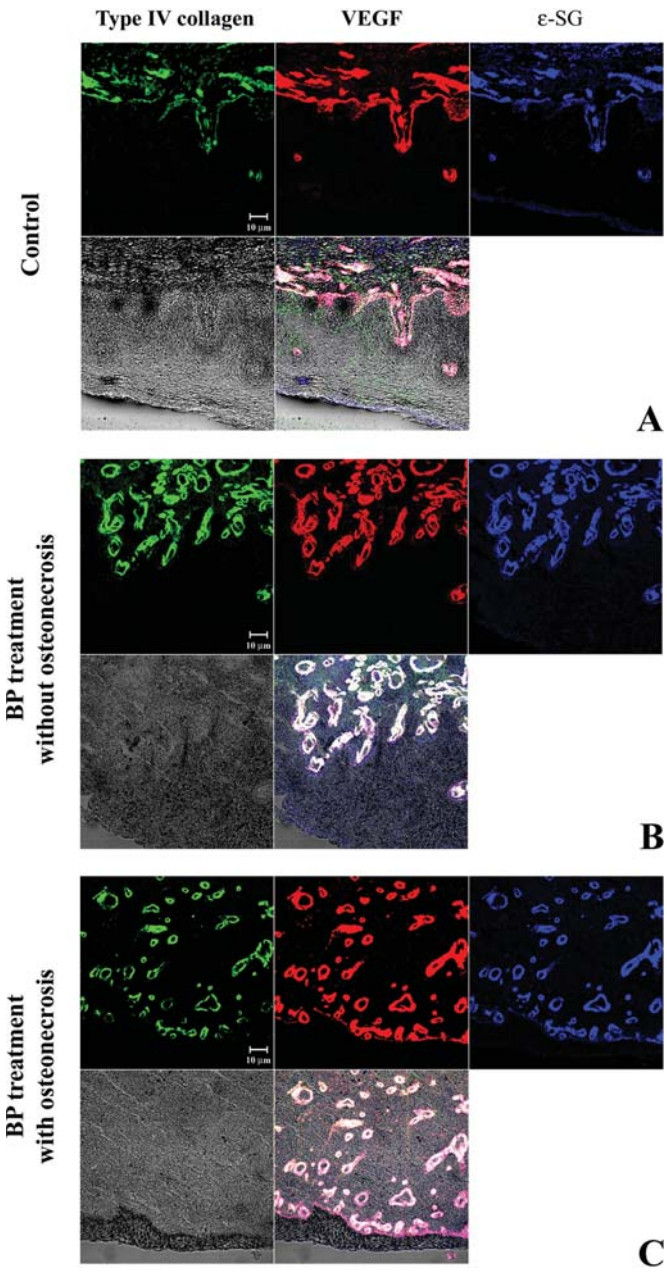

Figure 1. Compound panel showing immunohistochemical findings in human gingival epithelium immunolabeled with a triple immunofluorescence reaction, with type IV collagen (green channel), VEGF (red channel) and ε-sarcoglycan (blue channel). The gingival epithelium was analyzed in control subjects (A), in subjects treated with BPs showing no ONJ (B), and in subjects treated with BPs who showed ONJ (C). For each reaction it is possible to analyze the single proteins separately, the transmitted light and the merge between all channels. Almost complete absence of tested proteins on basal lamina was observed in subjects without ONJ, and a new increase of the same proteins on basal lamina in subjects with ONJ. Furthermore, qualitatively and quantitatively increasing neoangiogenesis was evident below the basal lamina.

1:50, anti-ßD-integrin diluted 1:50 (synthetic peptides from the COOH terminal region; kindly provided by the laboratory of Professor Tarone, University of Torino). In all reactions, TRITC-conjugated IgG anti-rabbit (red channel), FITC-conjugated IgG anti-mouse (green channel), and Texas Red-conjugated IgG anti-goat (blue channel), all from Jackson ImmunoResearch Laboratories, were used respectively. Slides were finally washed in PBS and sealed with mounting medium. To provide a control for non-specific immunostaining of sections we performed triple immunofluorescence reactions using secondary antibodies only.

The sections were then analyzed and images acquired using a Zeiss LSM 5 DUO confocal laser scanning microscope by META module. All images were digitalized at a resolution of 8 bits into an array of 2048x2048 pixels. Optical sections of fluorescent specimens were obtained using a HeNe laser (wavelength = 543 nm) and an Argon laser (wavelength = 458 nm) at a 1-min 2-sec scanning speed with up to 8 averages; 1.50 μm-thick sections were obtained using a pinhole of 250. Contrast and brightness were established by examining the most brightly labelled pixels and choosing the settings that allowed clear visualization of the structural details while keeping the pixel intensity at its highest (~200). Each image was acquired within 62 sec, in order to minimize photodegradation. Digital images were cropped and the figure montage prepared using Adobe Photoshop 7.0.

Results

To design a targeting model to better define the role of sarcoglycans, integrins, and matrix proteins in oral mucosa, we analyzed the immunofluorescence of these proteins in samples treated with bisphosphonates of subjects that did not show osteonecrosis; secondary, we studied samples of oral mucosa in patients affected by ONJ. Each group was, finally, compared with control samples.

In order to also study the colocalization of tested proteins, we performed triple immunofluorescence reactions; then, in all observations we applied the 'split' function, that permits to analyze the three fluorescences and transmitted light, separately, and the merge between all channels.

The triple immunofluorescence reaction, performed between type IV collagen (green channel), VEGF (red channel), and ε-sarcoglycan (blue channel) antibodies, in control subjects, showed a clear normal pattern of all proteins in basement membrane, which was perfectly delineated, and some vascular structures. The fluorescence analysis of the same proteins in samples treated with bisphosphonates, not pathological for ONJ, showed an almost absent protein staining pattern in the basal lamina. These proteins showed clearly detectable staining in the vascular structures. In the sections of subjects treated with bisphosphonates that showed osteonecrosis, we observed an increase of protein staining patterns on basal lamina and, contemporarily, qualitative and quantitative increase of vessels below the basal lamina (Fig. 1).

Triple fluorescence reaction performed with laminin (green channel), ß1D-integrin (red channel), and ß-sarcoglycan (blue channel) in control subject showed normal staining patterns of all proteins with a clear fluorescence on basal lamina. The same proteins in samples treated with bisphosphonates of subjects that showed no lesions, basal lamina fluorescence is absent whereas the vessels are clearly detectable. In the samples of subjects treated with bisphosphonates that showed osteonecrosis, it was possible to highlight new increase of staining patterns on basal lamina and a massive increase of vascular structures (Fig. 2).

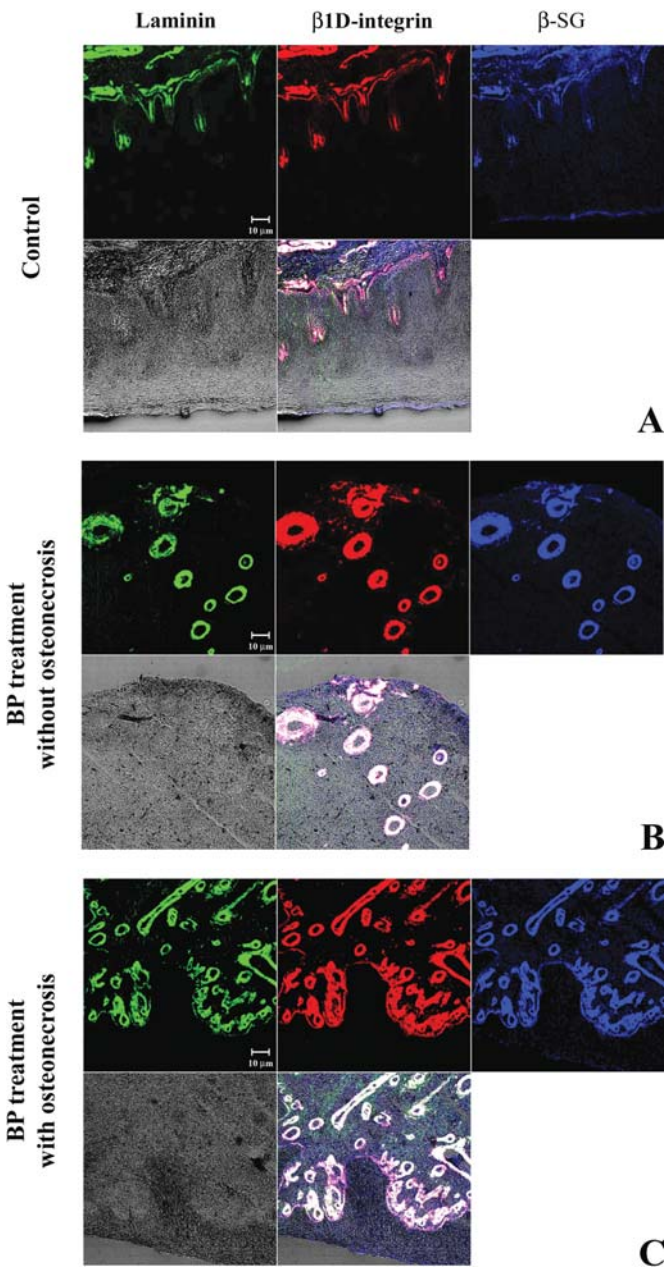

Figure 2. Compound panel showing immunohistochemical findings in human gingival epithelium immunolabeled by a triple immunofluorescence reaction, with laminin (green channel), ß1D-integrin (red channel) and ß-sarcoglycan (blue channel). The gingival epithelium was analyzed in control subjects (A), in subjects treated with BPs who showed no ONJ (B), and in subjects treated with BPs who showed ONJ (C). For each reaction it is possible to analyze the single proteins separately, the transmitted light and the merge between all channels. In subjects without ONJ, the almost complete absence of tested proteins in the basal lamina with contemporary increase of vessels was seen. In subjects with ONJ, a new increase of the same proteins on basal lamina and a further qualitatively and quantitatively increasing neoangiogenesis below the basal lamina were visible.

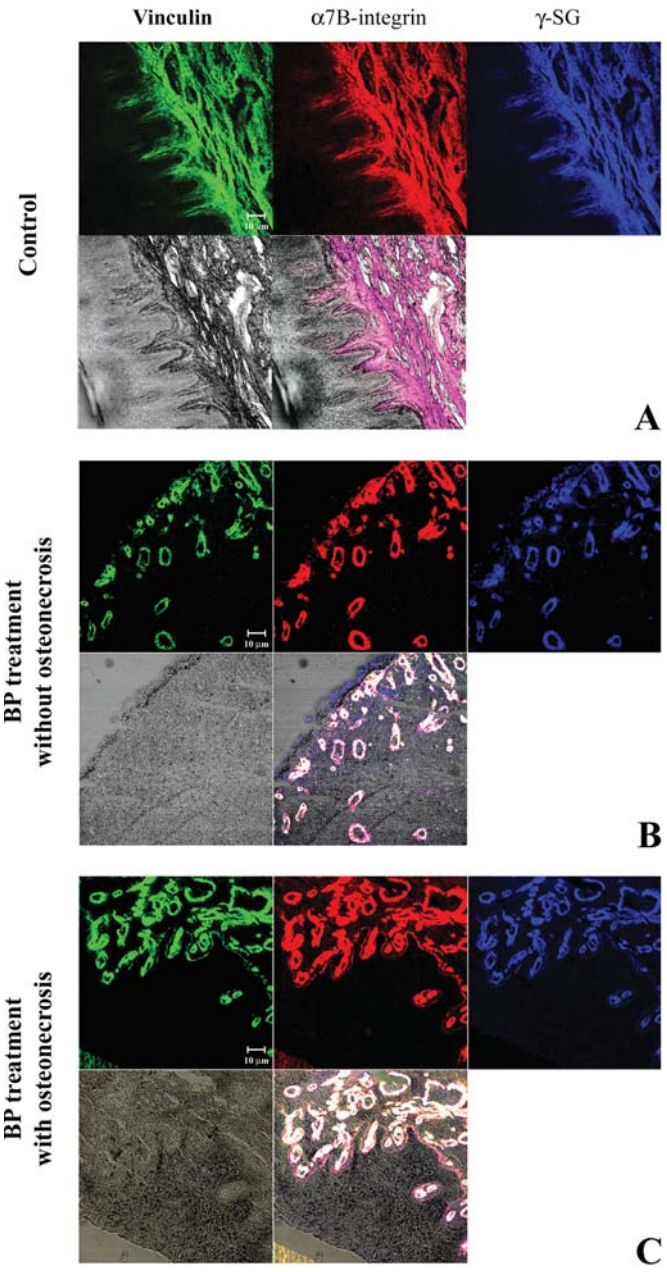

Figure 3. Compound panel showing immunohistochemical findings in human gingival epithelium immunolabeled by a triple immunofluorescence reaction, with vinculin (green channel), α7B-integrin (red channel) and γ-sarcoglycan (blue channel). The gingival epithelium was analyzed in control subjects (A), in subjects treated with BPs who showed no ONJ (B), and in subjects treated with BPs who showed ONJ (C). For each reaction it is possible to analyze the single proteins separately, the transmitted light and the merge between all channels. Our results showed in subjects without ONJ, an absence of tested proteins in the basal lamina, while the vascular structures appeared increased. In subjects with ONJ, an increase of the same proteins on basal lamina and a massive increase of neoangiogenesis below the basal lamina were observed.

Triple fluorescence reactions using vinculin (green channel), α7B-integrin (red channel), and γ-sarcoglycan (blue channel) antibodies showed the same behaviour of other proteins. In particular, in the control sample, a clear staining pattern of all proteins is detectable in the basal lamina, and a decrease, near absence, of the same proteins in basal lamina of samples treated with bisphosphonates without lesion with increase of vascular structures; finally, in the samples treated with bisphosphonates showing lesions, an increase of staining pattern both in the basal lamina and in vessels was detectable (Fig. 3).

Discussion

The basement membrane is a dynamic structure that undergoes quantitative and qualitative changes during diseases, being continuously remodelled by glycoprotein rupture and synthesis. These processes are important in inflammation and tissue repair, as the membrane becomes fragmented to allow inflammatory cell entry and exit. Such fragmentation needs to be orderly, rapidly and accurately repaired.

It is more than a support structure, and it is also involved in signalling during angiogenesis, since various angiogenesis-modulating molecules, growth factors and cytokines are stored in the basal membrane to be released and activated when the basal membrane is breached (32). An emerging paradigm is that the architecture itself transmits information to cells, through mechanisms such as matrix rigidity, spatial arrangement of cell receptors, and tension exerted between matrix and receptor (33,34). The adverse effects of BPs on oral epithelium may play a critical role in the onset of ONJ.

Landesberg *et al* (35) proposed that oral epithelial cells are subjected to local increases in BPs concentration after a traumatic event, and that the presence of BPs may inhibit epithelial wound healing, contributing to persistent exposure of underlying bone and development of ONJ. BP pre-treatment of oral mucosal cells inhibits proliferation and this inhibition is not due to cellular apoptosis (35).

Although numerous studies have been carried out on the behaviour of these proteins in most tissues, scarce data exist on their expression on oral mucosa in normal and in pathological conditions. However, it is well known that cells with disrupted talin function fail to form focal adhesions and exhibit spreading defects, whereas cells with vinculin disruption can form focal adhesions, but display reduced ability to spread and increase cell motility (36,37).

Here we used immunofluorescence techniques to analyze any proteins of vinculin-talin-integrin system and DGC, and moreover type IV collagen and laminin, in oral mucosa treated with bisphosphonates to evidence their role in these conditions.

In the present study, analyzing human gingival mucosa of subjects in treatment with bisphosphonates, for the first time, our data showed, clearly, that all tested proteins are almost absent, in basal lamina and oral mucosa of subjects treated with BPs but without osteonecrosis, whereas in oral mucosa with necrosis, patients showed a clearly detectable staining pattern of the same proteins, specifically in basal lamina, but less in comparison to control samples.

Moreover, by VEGF immunofluorescence analysis, in oral mucosa with lesion, a massive neoangiogenesis was clearly detectable. This is an unexpected finding as in previous studies we demonstrated the possibility that BPs could have a suppressive effect on angiogenesis (4,38). However, BPs could exert a different action on bone and mucosal tissue.

These data, for the first time, demonstrated that the tested proteins showed a key role in cellular signalling between cell and extracellular matrix. In particular, the increase of these proteins in basal lamina, concomitant with formation of the lesion, could indicate a compensative behaviour in the remodelling of the gingival mucosa in order to restore the epithelial architecture, and then to resume the signalling pathway of the cells. The neoangiogenesis, high-lighted by an increase of immunostaining patterns of two tested integrins and of VEGF, showed an unexpected feature during bisphosphonate treatment, confirming this compensative role of tested proteins.

Additional experiments performed to better clarify the action of BPs on oral mucosa should be made as ONJ lesion may initiate in the oral mucosa, and it is well known that human gingival fibroblast and human periodontal ligament cells might also have a role in osteoclastogenesis though the expression of receptor activator of nuclear factor kappa B ligand (RANKL) on their cell surface (39). Moreover, impact of BPs on mucosal cells could be useful to chose the most appropriate therapy as it was demonstrated that Pamidronate is not able to promote death of oral mucosal cells, although at BP doses greater than 0.1 mM there is a toxic effect that results in the detachment of the adherent cells.

Further studies on mucosal damage by BPs could indicate new paths of investigation leading to an improved understanding of ONJ.

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