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Bisphosphonates enhance bacterial adhesion and biofilm formation on bone hydroxyapatite

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ABSTRACT

Background: Because of the suspicion that bisphosphonates enhance bacterial colonization, this study evaluated adhesion and biofilm formation by *Streptococcus mutans* 25175, *Staphylococcus aureus 6538, and Pseudomonas aeruginosa* 14454 reference strains on hydroxyapatite coated with clodronate, pamidronate, or zoledronate.
Material and methods: Bacterial strains were cultured on bisphosphonate-coated and noncoated hydroxyapatite discs. After incubation, nonadhered bacteria were removed by centrifugation. Biofilm formation was confirmed by scanning electron microscopy. Bacterial colonization was estimated using quantitative cultures compared by means with Kruskal–Wallis and post-hoc Student-Newman-Keuls tests. Modeling of the interactions between bisphosphonates and hydroxyapatite was performed using the Density Functional Theory method.
Results: Bacterial colonization of the hydroxyapatite discs was significantly higher for all tested strains in the presence of bisphosphonates vs. controls. Adherence in the presence of pamidronate was higher than with other bisphosphonates. Density Functional Theory analysis showed that the protonated amine group of pamidronate, which are not present in clodronate or zoledronate, forms two additional hydrogen bonds with hydroxyapatite. Moreover, the reactive cationic amino group of pamidronate may attract bacteria by direct electrostatic interaction.
Conclusion: Increased bacterial adhesion and biofilm formation can promote osteomyelitis, cause failure of dental implants or bisphosphonate-coated joint prostheses, and complicate bone surgery in patients on bisphosphonates.

1. Introduction

The introduction of bisphosphonates (BPs) into clinical practice has considerably reduced the number of pathological fractures and metastases to bones, and has diminished the risk of hypercalcemia in oncologic patients. BPs have been quickly applied to prevent bone loss in osteoporosis, to treat bone dysplasia and metabolic bone diseases, and to improve the stability of endoprostheses (Chapurlat et al., 2004; Drake et al., 2008; Graham and Russell, 2011; Wilkinson and Little, 2011). Some of the adverse effects of using BPs were known from the beginning, such as increased temperature, atrial fibrillation, irritation of the digestive tract, and toxic effects on the kidneys (Drake et al., 2008).

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Many years later, additional complications associated with the use of the BPs were recognized, such as atypical fractures of the femur shaft and necrosis and inflammation of the jaw bone (Marx, 2003; Ruggiero et al., 2004; Isaacs et al., 2010; Thompson et al., 2012). Necrosis of large and therapy-resistant jaw bone exposures in patients receiving BPs is termed bisphosphonate-related osteonecrosis of the jaw (BRONJ). The pathogenesis of BRONJ still draws the attention of scientists, because many aspects of this phenomenon have not yet been explained.

Marx (2003) hypothesized that the inhibition of bone metabolism plays an important role; however, some pathological issues still cannot be explained on this basis. It is still not clear why necrosis of bone affiliated with the use of BPs develops only in the area of the jaws, or why the vast majority of cases result from exposure of the jaw bones following tooth extractions, oral surgery, pressure from poorly fitting dentures, or periodontitis, or why the disease is characterized by a high recurrence rate (Hasegawa et al., 2013; Barba-Recreo et al., 2014).

In trying to explain these problems, BPs may adversely affect wound healing through toxic actions on epithelial cells in addition to inhibiting bone metabolism (Acil et al., 2012), lowering the pH of the environment (Otto et al., 2010) and inhibiting angiogenesis (Stresing et al., 2011). At the same time, infection of the denuded bone with creation of biofilm composed of Gram-positive and Gram-negative strains as well as anaerobes has been documented in pathological and microbiological reports concerning BRONJ (Sedghizadeh et al., 2008; Wei et al., 2012). Moreover, microbial colonization in BRONJ cases was also significantly higher than in bone necrosis occurring in the absence of BPs, raising the possibility that BPs could enhance bacterial colonization and biofilm formation in patients treated with these drugs (Kos et al., 2010). In a recent report, we demonstrated that one of the BPs, namely, pamidronate, increases the adhesion to hydroxyapatite (HA) and facilitates the colonization of clinical and reference strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, species frequently associated with bone infections (Kos et al., 2013).

To investigate this characteristic in a broader group of BPs, we compared the adhesion and growth of Gram-positive and Gram-negative bacterial strains on hydroxyapatite discs coated with commonly used BPs including clodronate, pamidronate, and zoledronate, and we compared the results with those obtained with noncoated discs. Moreover, we applied molecular modeling based on the Density Functional Theory method to visualize the structure of the coated HA to explain the interactions of the hydroxyapatite surface with BPs as well as with bacterial receptors (Rimola et al., 2008).

The results of this study may help to answer questions about the potential risk of BP-linked bacterial adhesion causing infection after tooth extraction or in open fractures, osteotomies, or dental implants in patients on BPs, as well as the safety of using BPs to improve stability in joint arthroplasty.

2. Material and methods

2.1. Bacterial strains

For experimental purposes, the following ATCC reference strains were used: *Streptococcus mutans* 25175, *Staphylococcus aureus 6538*, and *Pseudomonas aeruginosa* 14454. Biofilms of *S. aureus* and *P. aeruginosa* were incubated in liquid Tryptic Soya Broth medium, whereas *S. mutans* was incubated in liquid Brain–Heart Infusion medium. These strains are part of the strain collection of the Department of Microbiology of the Medical University of Wroclaw.

2.2. Hydroxyapatite discs

The HA discs used for the experiments had the following characteristics: 0.38-inch diameter, 0.06- to 0.02-inch thickness, and purity greater than 95%; X-ray diffraction patterns that conformed to Joint Committee on Powder Diffraction Standard 9-432 (International Centre for Diffraction Data, Newtown Square, PA); total heavy metal as lead less than 40 ppm; and a trace element concentration that conformed to ASTM standard F1185-88 (ASTM International, West Conshohocken, PA).

2.3. Bisphosphates

The following BPs were used for experimental purposes: disodium clodronate (Bonefos; Bayer Pharma), disodium pamidronate (pamidronate; Medac) and zoledronic acid (Vipharm SA). The controls consisted of culture medium with 0.9% saline solution.

2.4. Coating of hydroxyapatite discs with bisphosphonates

The HA discs were coated with 200-μL aliquots of BPs, reaching a final concentration of 0.8 mg/L per disc. This concentration provides full saturation of the hydroxyapatite discs, which was tested in the series of preliminary experiments and our previous works (Kos et al., 2013; Junka et al., 2015). The HA discs were dried under a controlled, constant, heated airflow between applications. The presence of the coating was confirmed using a Zeiss EVO MA25 scanning electron microscope (Carl Zeiss Poland, Poland) with an energy dispersive X-ray microanalysis attachment and an accelerating voltage of 20 kV.

2.5. Confirmation by scanning electron microscopy of the investigated strains ability to form biofilms on hydroxyapatite discs

The *S. aureus* and *P. aeruginosa* strains, which were cultured on appropriate agar plates, were transferred to liquid media and incubated at 37 °C for 24 h under aerobic conditions. *S. mutans* was incubated at the same temperature and time, but in facultatively anaerobic conditions. After incubation, the densities of the bacterial suspensions were measured using a densitometer (Biomerieux Poland, Warsaw Poland) and diluted to 10^5 cells/mL. The bacterial dilutions were incubated in the presence of the HA discs at 37 °C for 24 h. After incubation, the discs were thoroughly rinsed using physiologic saline to remove nonadherent bacteria and to leave only biofilm-forming microorganisms. Subsequently, HA discs with biofilm on it were fixed using 3% glutarate (Poch, Gliwice, Poland) for 15 min at room temperature. Then, the samples were rinsed twice with phosphate buffer (Sigma Aldrich Poland, Poznan, Poland) to remove the fixative. The next step was dehydration in increasing concentrations of ethanol (25%, 60%, 95%, and 100%) for 5 min in each solution. After rinsing off the ethanol, the samples were dried. Then, the samples were covered with gold and palladium (60:40; sputter current, 40 mA; sputter time, 50 s) using a Quorum machine (Quorum International, Fort Worth, TX) and examined under a Zeiss EVO MA25 scanning electron microscope.

2.6. X-ray computed microtomography

To estimate the exact surface of the HA discs and to exclude the possibility that the differences in disc surface influence the results, computed microtomography methods (μCT) were used. Scaffolds were scanned using the μCT system (Metrotom 1500; Carl Zeiss, Oberkochen, Germany). The system consisted of a flat panel detector with a resolution of 1024 × 1024 px (400-μm pixel size) and a 16-bit grayscale, rotary table, and microfocus x-ray tube with a