Journal of Cranio-Maxillo-Facial Surgery 40 (2012) e229–e235

Contents lists available at SciVerse ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com

The cytotoxic effects of three different bisphosphonates in-vitro on human gingival fibroblasts, osteoblasts and osteogenic sarcoma cells

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ARTICLE INFO

Article history:
Paper received 31 May 2011
Accepted 10 October 2011

Keywords:
Osteonecrosis
Bisphosphonates
In-vitro
Human osteoblasts
Gingival fibroblasts
Osteogenic sarcoma cells

ABSTRACT

Introduction: Osteonecrosis of the jaw (ONJ) is an emerging condition in patients undergoing long-term administration of bisphosphonates (BP) for the treatment of osteoporosis and hypercalcaemia associated with malignancy, multiple myeloma, and metastatic breast and prostate cancers. This is a follow-up study, its purpose was to examine the effects in-vitro of intravenous zoledronic acid (ZOL) and pamidronate (PAM) and oral alendronate (FOS) on the human oral cavity using gingival fibroblasts and osteoblasts cells and, in addition, osteogenic sarcoma cells (SaOS-2-cells).
Materials and methods: Human gingival fibroblasts, osteoblasts and SaOS-2-cells were seeded on multiple 6-well plates at a density of 5×10^5 cells in a 4-week cell culture. Four different concentrations (1, 5, 10, 20 μM) of each BP (ZOL, PAM, FOS) and pyrophosphate were used in this study.
Results: All BP decreased collagen production and lowered cell proliferation in-vitro. ZOL was the component with most inhibitory effect.
Conclusion: The findings in this study suggest that ZOL, PAM and FOS generally diminish cell proliferation and collagen production of human gingival fibroblasts, osteoblasts and SaOS-2-cells. The present follow-up study shows that not only ZOL and PAM but also FOS have a strong inhibitory effect on collagen production and cell survival in-vitro.

1. Introduction

Osteonecrosis of the jaw (ONJ) is a recently emerging condition in patients undergoing long-term administration of bisphosphonates for the treatment of osteoporosis and hypercalcaemia associated with malignancy, multiple myeloma, metastatic breast and prostate cancers (Hortobagyi et al., 1996; Coleman, 2001; Saad et al., 2002; Santini et al., 2006; Abu-Id et al., 2008; Cafro et al., 2008; Matsumoto et al., 2009). The incidence of ONJ is not high, but it is very refractory to ordinary dental treatments, and the bone exposure, a typical symptom, continues for several weeks to months (Ruggiero et al., 2004; AAOMS, 2007; Junquera et al., 2009). Although many cases of ONJ have been reported worldwide, the precise pathogenesis remains obscure.

Bisphosphonates (BP) have two key properties: (1) strong binding to bone due to a high affinity for hydroxyapatite (Nancollas et al., 2006; Russell, 2006) and (2) the ability to inhibit osteoclast function (Luckman et al., 1998). The BP are analogues of pyrophosphate, in which the P–O–P bond of pyrophosphate is replaced by a P–C–P bond, which is highly resistant to chemical and enzymatic hydrolysis. The R1 and R2 side chains (containing e.g. nitrogen or non-nitrogen atoms) attached to the carbon are responsible for the variation in activity observed among these drugs (Fleisch, 1998). Their purpose is to inhibit bone reabsorption and increase bone mineral density. Consequently, BP are used in patients with bone dysfunction.

ONJ is a relatively new complication of supportive care in cancer and osteoporosis treatment. Bisphosphonate-associated ONJ can generally be defined as necrotic bone exposure to the oral cavity and inflammatory reactions of the surrounding soft tissue in patients receiving BP but not radiotherapy to the head and neck. Various clinical reports have been made with precise descriptions since 2003 (Marx, 2003; Ruggiero et al., 2004; Ficarra et al., 2005; Marx et al., 2005).

The risk of developing ONJ varies with the type of BP used and the duration of exposure, with more potent agents increasing the risk with shorter durations of exposure. Zoledronic acid is the most potent bisphosphonate (Fleisch, 1998). The most frequently used BP and those responsible for ONJ are zoledronic acid (ZOL) and pamidronate (PAM), which are administrated intravenously and are

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1010-5182/$ – see front matter © 2011 European Association for Cranio-Maxillo-Facial Surgery.
doi:10.1016/j.jcms.2011.10.024

given for malignant osteolytic lesions (Purcell and Boyd, 2005; Cafro et al., 2008). But numerous cases of ONJ have been reported in patients taking oral BP, mainly in the treatment of osteoporosis (Ruggiero et al., 2004; Hong et al., 2010; Yarom et al., 2010). The medications used in these cases are mostly alendronate, ibandronate and risedronate, with alendronate (FOS) being frequently reported to cause adverse reactions such as ONJ (Bocanegra-Pérez et al., 2009; Sedghizadeh et al., 2009).

The limited data on specific reactions of oral and intravenously administered BP have lead to this follow-up in-vitro study. A previous study examined the effects of two intravenous BP (Simon et al., 2010). The purpose of this follow-up study was to examine the effects of ZOL, PAM and FOS and possibly identify the highest tolerable concentration on the human oral cavity, represented by gingival fibroblasts and osteoblasts cells, and osteogenic sarcoma cells (SaOS-2-cells), to see its potential effect on cells with a higher turn-over rate, as well as identifying probable differences between these three different drugs and possibly confirm previously published data. The selected cells were incubated with BP for 4 weeks. Following this the cells response to the exposure to BP was analyzed with cell proliferation tests, ELISA and HPLC.

2. Materials and methods

2.1. Human cell culture

Human osteoblasts and fibroblasts were isolated from samples of the iliac crest and of the gingiva, respectively. The osteoblast samples were obtained from healthy patients who had no exposure to BP therapy, undergoing reconstruction surgery for cleft lip and palate from our department of oral and maxillofacial surgery. All patients had been informed about this study and had signed a letter of informed consent (Ethic number: D 402/07). Preparation of the tissue samples and further processing was performed as previously described (Acil et al., 2000, 2002). Human osteogenic sarcoma cells (SaOS-2-cells) were purchased from DSZM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Spongiosa cells of the sample and SaOS-2-cells were defined as osteoblast-like cells by the determination of osteoblast markers (biosynthesis of osteocalcin and activity of alkaline phosphatase). Fibroblasts from gingival samples were obtained during routine surgical procedures. The gingival fibroblasts were tested and exhibited the typical morphologic fibroblast patterns. The tests for osteocalcin and alkaline phosphatase were negative, as described previously (Acil et al., 2000, 2002).

The cells were subcultured and transferred as Simon et al. described (Simon et al., 2010). Each cell-line was seeded on multiple 6-well plates at a density of 5×10^5 cells.

2.2. Alkaline phosphatase (ALP) activity

ALP activity was determined by cytochemistry with Sigma Diagnostic Kit (86-R, Deisenhofen, Germany), as described previously (Acil et al., 2000, 2002).

2.3. Osteocalcin synthesis

The expression of osteocalcin was studied using monoclonal antibodies provided by Takara (Takara Shuzo, Co., Ltd., Japan), as described by Acil and colleagues (Acil et al., 2000, 2002).

2.4. Bisphosphonates (BP)

In order to relate the scientific research to clinical processes, the decision was made to use the most common drugs associated with ONJ (Ramaswamy and Shapiro, 2003; Ruggiero et al., 2004; Ficarra et al., 2005; Marx et al., 2005; Kumar et al., 2008; Sedghizadeh et al., 2009); Zoledronic acid (ZOL; Zometa®, Novartis, 200 mg, i.v., Nürnberg, Germany) and pamidronate (PAM; Aredia®, Novartis, 506 mg, i.v., Nürnberg, Germany); alendronate sodium (FOS; Fosamax®, MSD Sharp & Dohme GmbH 85530 Haar, Germany) plus inorganic pyrophosphate (PP; techn., Aldrich, Taufkirchen, Germany) as a positive control component. Four different concentrations (1, 5, 10, 20 μM) of each molecular compound were elected and used in this study.

2.5. 4-week cell culture

After seeding of the cells (at 5×10^5 cells per well) of each cell-line, they were cultured in ten groups of 6-well plates, each with different concentrations of supplemented growth medium, for 4 weeks. Every third day, the medium was renewed with the three different substrates (ZOL, PAM, FOS, PP) and with the specific concentrations. The used growth medium from the cell cultures was saved and pooled from every transfer for further testing. The experiment was terminated on day 28, and analysis was performed.

2.6. Cell proliferation test (MTT)

Additionally to the 6-well plates, cell cultures with the BP and their particular concentrations were incubated on 96-well plates for 7 days in ten-fold. The cell proliferation was evaluated with the Cell Proliferation Kit I (MTT, Roche Diagnostics GmbH, Penzberg, Germany) on day 1 and day 7. The Cell Proliferation Kit I (MTT) measures the metabolic active and viable cells. The reaction produced a water-insoluble formazan salt that was solubilized and ultimately assessed in a flow through spectrometer, as described previously (Simon et al., 2010).

2.7. Enzyme-linked immunosorbent assay (ELISA)

After each week 100 μL cell culture medium from each sample of the cells and with their specific supplemented concentrations of the 4-week cell culture were collected and further used in the standardized ELISA. The following assay was performed for collagen type I and it was finalized with a flow through spectrometer, as described previously (Simon et al., 2010).

2.8. Preparing for SDS-PAGE and HPLC

At the end of the 4-week cell cultures, sample volumes of the collected used medium were determined. The sample volumes being 80 mL, 5 N NaCl were added and a stirring process at room temperature over a minimum of 12 h began. Following a centrifugation, in Falcon-tubes at 8000 U/min for 60 min at 4 °C (Centrifuge Sigma, Deisenhofen, Germany) the pellets were resuspended in 6 mL 0.5% acetic acid + 0.2 M NaCl. Next, an ultracentrifugation was performed using VivaSpin-tubes (VivaSpin 6, VivaScienceAG, Sartorius Group, Hannover, Germany) at 8000 U/min at 4 °C for approximately 2 h, the remaining pellet was resuspended twice in 3 mL 0.5% acetic acid + 0.2 M NaCl and made soluble. The solution's pH level was lowered to 2 for a 24 h pepsin digestion, 0.2 mg/mL (Pepsin porcine, Serva Electrophoresis GmbH, Heidelberg, Germany). Thereafter a repeated ultracentrifugation in VivaSpin-tubes followed, under the same conditions as described above, and the pellet was resuspended four times with 2.5 mL 0.5% acetic acid. Two and a half millilitres of the suspension were used to do a SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) and the other 7.5 mL were needed to perform hydrolysis in order to prepare CF-1 to gain quantification