UNIVERSITÀ DEGLI STUDI DI TORINO

Preventive oral surgery before bisphosphonate administration to reduce osteonecrosis of the jaws

Short title: Preventive oral surgery and ONJ by bisphosphonates

Key words: Bisphosphonates, osteonecrosis, jaws, cytokines, RANK-L, HMGR

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Abstract

Objectives

The intravenous injection of bisphosphonates, currently used for osteoporosis, myeloma or bone metastases, can cause ONJ especially in consequence of trauma. In order to avoid trauma during bisphosphonate treatment, preventive oral surgery is recommended.

The research aimed to evidence whether inflammatory and osteoclastogenic factors are not induced in oral mucosa after bisphosphonate treatment in patients receiving oral preventive surgery procedure, and whether proliferation factors are not inhibited.

Patients and Methods

Specimens of oral mucosa were removed from healthy subjects and from patients undergoing preventive oral surgery before bisphosphonate treatment. The expression of cytokines, factors involved in osteoclast activity, in cell proliferation and angiogenesis were examined.

Results

Cytokines and RANK-L levels decreased significantly in mucosa from patients undergoing preventive oral surgery procedure before bisphosphonate treatment in comparison with their levels at the beginning of procedure and also in comparison with the level in patients treated only with bisphosphonates and not developing ONJ; conversely, osteoprotegerin and hydroxymethylglutaryl coenzyme A reductase significantly increased or not changed.

Conclusions

The results suggest that preventive oral surgery could be able to prevent ONJ due to bisphosphonate treatment: the mucosa is not stimulated by bisphosphonates to cause ONJ, since bisphosphonates are probably not released from the bone.

Introduction

Bisphosphonates (BP) are used in treating patients bearing solid tumors or myelomas, to reduce skeletal alterations due to bone metastasis. They are thus increasingly incorporated in oncologic treatments aimed at improving the quality of life. In 2003, an association between BP use and osteonecrosis of the jaw (ONJ) in oncologic patients was reported (Brotons and Penarrocha, 2003). The American Association of Oral and Maxillofacial Surgeons (AAOMS) reported that the incidence of ONJ in patients treated with BP in whom this therapy is associated with dental extractions and/or oral surgery procedures ranges from 0.8 to 12% (Advisory Task Force on Bisphosphonate-Related Ostenonecrosis of the Jaws, 2007; Marx, 2007). ONJ is a chronic complication of BP therapy that chiefly occurs when administration is intravenous; the highest frequency is in the mandible (Marx, 2003; Wang et al., 2003; Ruggiero et al., 2004).

In the light of these observations, dental management protocols to be implemented before BP administration may be crucial, in order to avoid oral intervention during or after BP treatment. Such protocols, eliminating any potential infection sites in patients before BP treatment, aim to ensure an adequate oral health status, in order to reduce the need for tooth extraction, and as a consequence, the risk of ONJ (Advisory Task Force on Bisphosphonate-Related Ostenonecrosis of the Jaws, 2007; Rayman et al., 2009). Studies on preventive dental measures have demonstrated a decreased incidence of ONJ in patients treated with BP for bone metastases from solid tumors (Ripamonti et al., 2009).

The mechanisms underlying ONJ are not fully understood. Recent studies have suggested involvement of the mucosa in this syndrome (Mozzati et al., 2013; Morris and Cruickshank, 2010; Silverman and Landesberg, 2009). In patients treated for at least 12 months with zoledronic acid, a third-generation BP containing nitrogen, the mucosa produced inflammatory factors and factors stimulating osteoclasts, in those cases in which ONJ subsequently developed (Mozzati et al., 2013). The possible involvement of the mucosa was confirmed using an “in vitro” model, since the ”in vivo” observations did not fully elucidate the interactions between soft-tissues and bone that lead to ONJ (Saracino et al., 2012). The “in vitro” model consisted of epithelial cells exposed to zoledronic acid, and osteoblasts grown in a culture medium conditioned by epithelial cells treated with zoledronic acid. The results showed that treatment of epithelial cells with zoledronic acid reduced cell proliferation, suggesting that this treatment may decrease cells’ healing ability after injury. Moreover, zoledronic acid increased the release in the culture medium of pro-inflammatory cytokines, such as TNFα, and decreased cell content of PPARα, a member of the nuclear hormone

receptor superfamily that plays a beneficial role in reducing inflammation. In turn, when this medium rich in pro-inflammatory cytokines was used to grow osteoblasts, it affected their proliferation, possibly reducing the regenerate capability of the bone; it also increased expression of RANK-L, which is a factor involved in stimulating osteoclast differentiation (Saracino et al., 2012).
In the light of the probable importance of epithelial cells in inducing ONJ after treatment with BP, and of the importance of adopting preventive measures before administration of these drugs, in order to avoid any trauma that may trigger ONJ, the present study examined the mucosa of patients before the preventive oral surgery and the administration of zoledronic acid, and after the intravenous administration of the BP.

Patients and Methods

Patients

Eleven patients (group A) with bone metastases from solid tumors or multiple myeloma, waiting for therapeutic treatment with intravenous zoledronic acid, and 11 healthy subjects, entered the study. The patients received 4 mg intravenous zoledronic acid (Zometa®; Novartis Pharma SpA, Basel, Switzerland) every 4 weeks for 12 months; they did not receive radiotherapy at the head and neck region, nor any chemotherapeutical medications during zoledronic acid treatment. Informed consent was obtained from all patients. The study protocol was approved by the Ethics Committee of Turin University (local ethical board approval number: CEI/396; protocol number: 0010469). Patients treated with BP for osteoporosis were excluded.
At their inclusion in the trial, the patients were registered in a computerized clinical file, which recorded information on age, sex, smoking habits, alcohol consumption, systemic pathologies and the use of any drugs. Each patients was subjected to a periodontal evalutation by PSR code (periodontal screening and recording): all patients had a PSR score between 0 and 2.
Afterwards, each patient attended a program of professional dental hygiene to nullify differences in preoperative hygiene conditions.
Before treatment with zoledronic acid, the patients of group A underwent an oral preventive surgery to remove any potential infection sites.
During zoledronic acid administration, all the patients were observed for the absence or presence of ONJ.

Oral Preventive Surgical Procedure

In the group A patients, extraction of only one tooth was performed under locoregional anaesthesia (3% mepivacaine with 1:100,000 epinephrine), and was performed atraumatically, without elevation of full-thickness flaps; healing was natural, by clot formation. In all cases, vycril 4/0 sutures were used to suture the soft tissue and removed after 7 days. No other preventive surgery was done. Patients were given antibiotic (amoxicillin every 8 hours for 6 days) and oral anti-inflammatory treatment (Ibuprofen every 12 hours for 3 days).

Mucosa specimens

Mucosa specimens were taken from an area corresponding to the maxillary protuberance, from healthy subjects, and from group A patients before tooth extraction (time A1) and one month after the completion of the treatment with zoledronic acid (time A2). Excision was half-thickness, to avoid removing the periosteum and exposing the bone. A simple suture was placed. All specimens were placed in RNA Later solution (Qiagen, Milan, Italy), and maintained at –80°C until use. The values obtained at A2 time have been compared with those obtained in patients who did not undergo preventive oral surgical procedure, treated with zoledronic acid, but without the presence of ONJ (ONJ-) (values previously published) (Mozzati et al., 2013).

Radiographic analysis

Patients were monitored through dental panoramic radiography and computed tomography (CT), to evaluate the presence or absence of ONJ.

Biological factor analysis

Mucosa specimens were analyzed to determine expression of inflammatory (IL-1β, IL-6, IL-8 and TNFα), osteoclastogenic (RANK-L and osteoprotegerin, OPG), proliferation (3-hydroxy-3-methyl-glutaryl-CoA reductase, HMGR) and angiogenetic (VEGF) parameters, using real-time polymerase chain reaction (PCR).

Total RNA was extracted from specimens using the NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), as reported elsewhere (Mozzati et al., 2011). Real-time PCR was run with single-stranded cDNA prepared from total RNA (1 µg) using a High-Capacity cDNA Archive kit (Applied Bio Systems, Foster City, CA).

The forward (FW) and reverse (RV) primers were designed using Beacon Designer® software (Bio-Rad, Hercules, CA). Twenty-five µl of a PCR mixture containing cDNA template equivalent to 40 ng of total RNA, 5 pmoles each of forward and reverse primers, and 2× IQ SYBR Green

SuperMix (Bio-Rad, Hercules, CA) were amplified using an iCycler PCR instrument (Bio-Rad, Hercules, CA) as previously reported (Mozzati et al., 2013). Each sample was tested in duplicate, and threshold cycle (Ct) values from each reaction were averaged. GAPDH was used as house-keeping gene. The changes in expression were defined as those detected in the mucosa specimens taken from patients at times A1 or A2, versus those detected in the mucosa specimens taken from healthy subjects, and were calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{T\ sample} - \Delta Ct_{Tnormal}$.

Statistical analysis

Statistical analysis was performed using the GraphPad Instat software package. For each biological factor examined, differences between the mean values of the two mucosa specimens, A1 and A2, taken before and after treatment with zoledronic acid, were assessed using the paired t-test. Differences between the mean values of specimens A2, and those of ONJ-, were assessed using the Mann-Whitney Test. Differences were taken as being statistically significant for $p<0.05$.

Results

Patients

Group A enrolled 11 patients: 7 men (64%) and 4 women (36%) with mean age of 58 ± 9 years. 5 patients had multiple myeloma (46%), 3 had prostatic carcinoma (27%) and 3 had lung carcinoma (27%). All patients underwent a surgical procedure to remove only one tooth. The BP treatment started 30 days after tooth extraction, when the mucosa of extraction socket was completely healed in all patients.

Radiographic analysis

The patients enrolled were monitored through dental panoramic radiography followed by the more informative CT imaging. This is in line with previous findings (Bianchi et al., 2007). Having undergone preventive oral surgery before zoledronic acid administration, no patient showed any signs of ONJ as evidenced by both dental panoramic radiography and CT imaging (data not shown).

Biological factor analysis

Expression of the biological factors involved in the inflammatory process, IL-1β, IL-6, IL-8 and TNFα, is shown in Figure 1. Levels of all cytokines varied in the mucosa specimens taken before versus those taken after administration of zoledronic acid, with the exception of TNFα, which was similar at the two times. Cytokine levels, referred to values found for healthy subjects taken as 1, were found to be lower in specimens taken after administration of zoledronic acid (time A2) than in those taken before zoledronic acid administration (time A1).

Figure 2 shows the variation of RANK-L and OPG between specimens of mucosa taken before and after administration of zoledronic acid. RANK-L was decreased in mucosa specimens taken after zoledronic acid administration versus those taken before administration. Conversely, OPG did not differ before and after zoledronic acid administration.

As regards factors involved in cell proliferation (HMGR) and in endothelial cell proliferation (VEGF), Figure 2 also shows that HMGR increased in the mucosa after zoledronic acid administration, versus mucosa taken before the preventive oral surgery and administration of zoledronic acid, whereas VEGF showed no variation..

Our previous research [9] investigated the same parameters in the mucosa taken after treatment with zoledronic acid from patients not undergoing the preventive oral surgery, who did or did not go on to develop ONJ. To confirm the beneficial effect of the preventive oral surgery, the values of the various parameters obtained from the mucosa of patients who had undergone the preventive surgery protocol followed by administration of zoledronic acid (time A2) were compared to the corresponding values obtained from the mucosa of patients treated with zoledronic acid without prior preventive oral surgery, and who showed no signs of ONJ (ONJ-). Figure 3 shows that all inflammatory parameters in mucosa from patients at timeA2, with the exception of TNFα, had values lower than those observed in the ONJ- patients. Similarly, Figure 4 shows that the values of parameters relating to osteoclast differentiation are lower in patients at time A2 in comparison with those of the ONJ- patients. As regards the angiogenesis and proliferation parameters, VEGF was lower in time A2 specimens than in ONJ- specimens, whereas HMGR values were unchanged.

Discussion

The study aimed to evaluate the beneficial effect of a preventive oral surgery in patients who are scheduled for treatment with BP, for bone metastases from solid tumors or for myeloma. The efficacy of BP has been demonstrated in clinical trials, in which they were administered by

intravenous injection (Pavlakis et al., 2005); Aapro et al., 2008). However, the use of BP in oncology is sometimes accompanied by an adverse effect, namely the development of ONJ. This side effect can negatively affect the patient's quality of life (La Verde et al., 2008; Ruggiero et al., 2006). ONJ may develop during invasive dental interventions (Vandone et al., 2011) or in an apparently spontaneous manner (Lo et al., 2010). It appears probable that trauma, even if minor, is always the cause of ONJ. Since no alternative therapy is currently available for patients with metastatic bone disease, a possible approach is to remove any potential infection sites in patients before BP treatment, in an effort to guarantee an adequate oral health status and reduce the risk of ONJ (Advisory Task Force on Bisphosphonate-Related Ostenonecrosis of the Jaws, 2007; Rayman et al., 2009). It was thus felt to be important to evaluate, alongside clinical and radiography observations, inflammatory, osteoclastogenic, angiogenesis and cell proliferation parameters, so as to verify the beneficial effects of a preventive oral surgery, applied prior to administration of zoledronic acid. Specimens of mucosa were taken from patients at two time-points, in order to identify any differences in gene expression before the preventive surgery and zoledronic acid treatment (time A1), and after 12 months of zoledronic acid treatment (time A2). Moreover, the results obtained at time A2 were also compared with specimens from patients treated with zoledronic acid who had not undergoing the preventive surgery protocol, and who did not go on to develop ONJ.

It is noteworthy that, at time A2, patients showed no signs of ONJ after 12 months of zoledronic acid treatment, and that lower levels of the inflammatory and osteoclastogenic factors were produced by the mucosa than at time A1. This decrease, or lack of change, in inflammatory factors is in contrast with findings that zoledronic acid causes an inflammatory acute-phase response (Russell et al., 1999); this response due to cytokine production is maximal within 28–36 h after intravenous administration, and disappears 2–3 days later, despite continuing treatment (Santini et al., 2007). The different results we obtained may be due to the fact that, in this study, specimens of mucosa were taken one month after the 12^{th} dose of zoledronic acid. Our results seem to suggest that the decreased IL-6 expression is correlated, as well as with a general decrease in the inflammatory response, also with the decreased osteoclast activity, as demonstrated by the decreased RANK-L expression. In fact, IL-6 is considered to be an osteoclastogenic cytokine, important in inducing bone loss (Rufo et al., 2011; Jimi et al., 2010). Moreover, it has been reported that induction of IL-6 due to pro-inflammatory stimuli is also accompanied by down-regulation of markers of osteoblastic differentiation (Kraus et al., 2011; Krishnan et al., 2011), and thus its decrease allows osteoblasts to engage in osteogenic action.

Unlike IL-6, HMGR expression did not change at time A2in comparison with time A1; this might be significant, because this enzyme is important in the synthesis of cholesterol and isoprenoids. Cholesterol at physiological concentrations is crucial for osteoblastic differentiation (Viccica et al., 2007), and isoprenoids are involved in the isoprenylation of some proteins, such as ras, involved in regulating cell proliferation (Cho and Lee, 2002). The accumulation of unprenylated proteins via downregulation of HMGR is believed to largely account for the cytotoxic effects of zoledronic acid Raikkonen J et al., 2010). In a previous paper we also reported that, in mucosa taken from patients with ONJ, the mRNA content of HMGR was lower than in that of the patients without ONJ (Mozzati et al., 2013). Moreover, the " in vitro" treatment of epithelial cells with zoledronic acid caused a decreased HMGR expression (Saracino et al., 20112).

The results reported here could partially explain why BP induce osteoclast activity and bone necrosis in the jaw, but not in other bones, where BP inhibit osteoclast activity in order to reduce bone loss: BPs are known to accumulate in the bone, and may be released from the bone into the surrounding tissues in response to minor traumas (dentures or dental prostheses, caries and periodontal disease) (Woo et al., 2006). In the case of the jaw, the tissue concerned is the mucosa, where epithelial cells, fibroblasts and lymphocytes are present. In this context, BP inhibit HMGR, and induce these cells to produce cytokines. If ONJ is to be prevented, such trauma must if at all possible be avoided; this can be facilitated by means of preventive surgical treatment to eliminate possible sources of infection.

The reduction of VEGF production in mucosa specimens after treatment with zoledronic acid, found here, could be explained by the fact that preventive surgical treatment is unable to prevent the antiangiogenic effect of BP, but this effect is not in itself enough to cause the development of ONJ. At the present, contrasting results have been reported about the effect of BP on angiogenesis, as it relates to ONJ development. (Landesberg et al., 2011; Rustemeyer and Bremerich, 2010).

The comparison between mucosa from patients treated with oral preventive surgery before zoledronic acid treatment, and mucosa from patients receiving zoledronic acid alone who did not develop ONJ, showed that cytokines and osteoclast-stimulating factor levels were lower in the former group than in the latter group. This confirms that preventive oral surgery before BP administration is a good practice.

In conclusion, preventive oral surgery could be able to prevent ONJ due to zoledronic acid treatment: the mucosa is not stimulated by BP to cause ONJ, since BP are probably not released from the bone, in consequence of lack of trauma after zoledronic acid treatment. The decrease in IL-6 leads to decreased osteoclastogenic activity, evidenced by the decreased RANK-L: increased

HMGR levels are conducive of good proliferative ability. Further studies with a long-term follow-up and with larger number of patients will be necessary to confirm the results of this study, that is that preventive oral surgery is fundamental to prevent ONJ.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1

mRNA content of IL-1β, IL-6, IL-8, TNFα in the mucosa taken from patients before preventive oral surgery (time A1) and after zoledronic acid administration (time A2)

The data are means ± S.D. of values obtained from the mucosa of 11 patients of group A, and are expressed as fold increase determined as described in the patients and methods section.

* paired t-test, $p<0.05$ time A2 versus time A1

Abbreviations: A1, time A1; A2, time A2

Figure 2

mRNA content of RANK-L, OPG, VEGF, HMGR in the mucosa taken from patients before preventive oral surgery (time A1) and after zoledronic acid administration (time A2)

The data are means ± S.D. of values obtained from mucosa of 11 patients of group A and are expressed as fold increase determined as in the patients and methods section.

* paired t-test, $p<0.05$ time A2 versus time A1

Abbreviations: A1, time A1; A2, time A2

Figure 3

mRNA content of IL-1β, IL-6, IL-8, TNFα in the mucosa taken from patients not receiving the preventive surgery treatment and free of ONJ after zoledronic acid administration (ONJ-), and from patients receiving preventive oral surgery followed by zoledronic acid administration (time A2)

The data are means ± S.D. of values obtained from mucosa of 16 patients for ONJ- and 11 patients for A group and are expressed as fold increase determined as described in the patients and methods section.

* Mann-Whitney Test, $p<0.05$ time A2versus ONJ-

Figure 4

mRNA content of RANK-L, OPG, VEGF, HMGR in the mucosa taken from patients not receiving preventive surgery treatment, and free of ONJ after zoledronic acid administration (ONJ-) and from patients receiving the preventive oral surgery followed by zoledronic acid administration (A2).

The data are means ± S.D. of values obtained from mucosa of 16 patients for ONJ- and 11 patients for A group and are expressed as fold increase determined as described in the patients and methods section.

* Mann-Whitney Test,$p<0.05$ time A2versus ONJ-

Figure 1

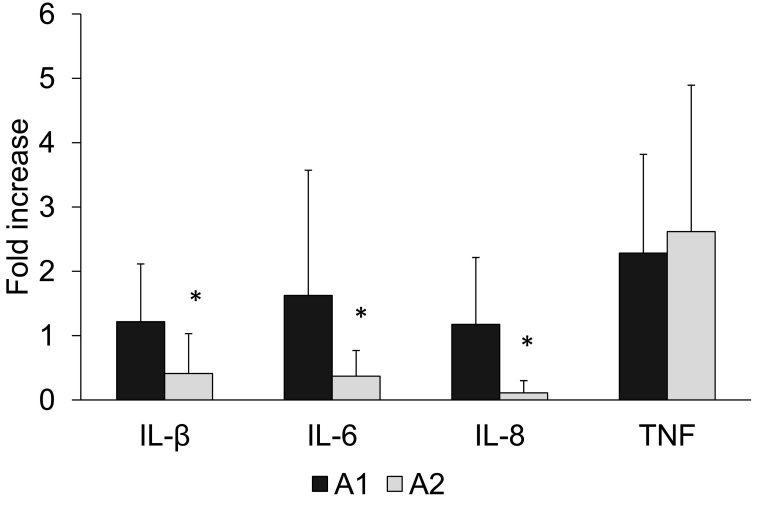

Figure 2

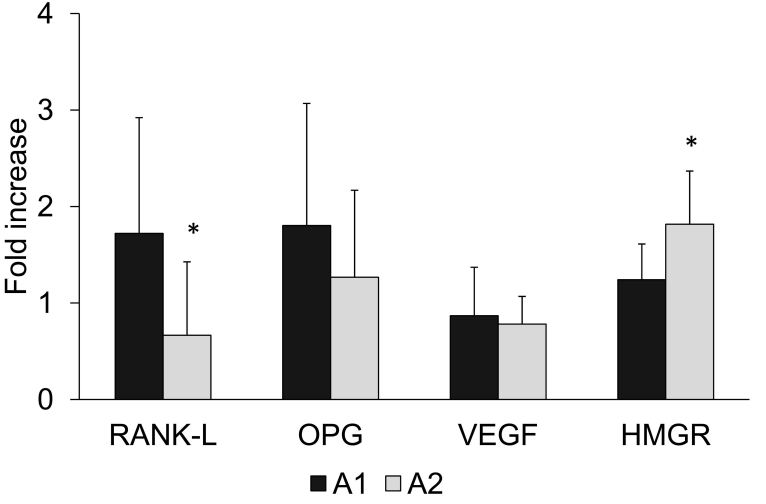

Figure 3

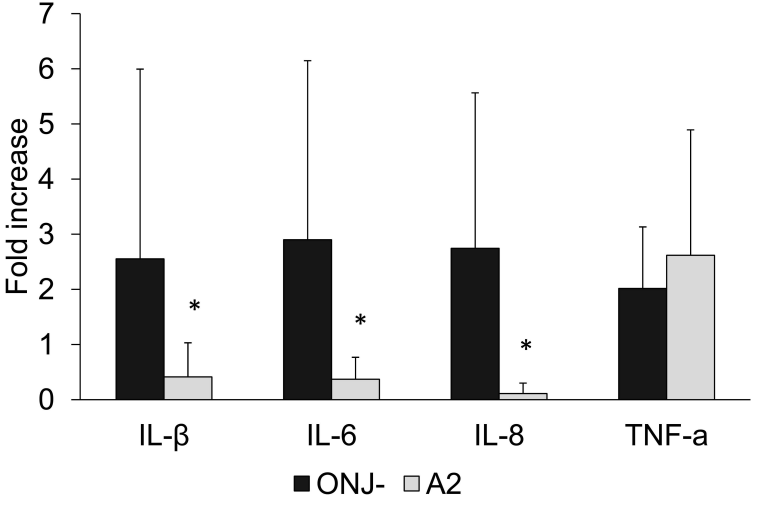

Figure 4