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In vitro cytotoxicity of zoledronate (nitrogen-containing bisphosphonate: NBP) and/or etidronate (non-NBP) in tumour cells and periodontal cells

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ARTICLE INFO

Article history:
Accepted 16 November 2012

Keywords:
Bisphosphonate
Cytotoxicity
Zoledronate
Etidronate

ABSTRACT

Objective: Nitrogen-containing bisphosphonates (NBPs), the first-choice drugs for diseases that cause enhanced bone resorption, may injure jawbones and gastrointestinal tissues. In rodents, NBPs cause necrosis at injection sites. Bisphosphonates accumulate within bones, especially where there is inflammation. We hypothesized that if jawbone-accumulated NBPs are released, they may directly injure cells around the jawbones. To examine this hypothesis, we compared the direct effects of zoledronate (NBP) and/or etidronate (non-NBP) on various cells, including periodontal cells.

Design: Various human tumour cells (such as squamous carcinoma cells and prostate adenocarcinoma cells) and periodontal cells (such as gingival fibroblasts and periodontal ligament cells) were incubated with or without zoledronate and/or etidronate. Cell viability and cytotoxicity were determined by tetrazolium dye assay and by FITC-Annexin V/propidium iodide assay, respectively.

Results: Zoledronate, at 100 μM, was toxic to all types of cells tested, while its toxicity varied among cells at both 1 and 10 μM. There was no clear difference between tumour cells and non-tumour cells in sensitivity to the cytotoxicity of zoledronate. In contrast, etidronate was not toxic at 1–100 μM in any of the cells tested. Interestingly, etidronate reduced the cytotoxicity of zoledronate in many cell-types, including gingival fibroblasts.

Conclusions: These results, together with those reported by others and those from our previous *in vivo* experiments, suggest that NBPs, upon release from jawbones (e.g., during dental surgery or bone infection), may directly injure various cells located around the jawbones, and that etidronate may be protective against the cytotoxicity of NBPs in periodontal tissues.

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Abbreviations: BPs, bisphosphonates; HCEM, human cementoblasts; HGF, human gingival fibroblasts; HGMS, human gingival mesenchymal stem cells; HPDL, human periodontal ligament cells; HUVEC, human umbilical vein endothelial cells; NBPs, nitrogen-containing bisphosphonates; PI, propidium iodide.

0003–9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.archoralbio.2012.11.010

1. Introduction

The anti-bone-resorptive activities of nitrogen-containing bisphosphonates (NBPs) are much more powerful than those of non-NBPs.[1,2] Thus, NBPs are the first-choice drugs for various diseases that cause enhanced bone resorption. However, NBPs may induce necrosis, with ensuing exposure of jawbones.[3,4] Zoledronate (approved in the USA for clinical use in 2001) is the NBP with the most potent anti-bone-resorptive effect,[2] but it also carries the highest risk of injury to jawbones.[4] It is also known that NBPs, when given orally, may directly injure esophageal and gastrointestinal tissues, resulting in esophagitis, nausea, vomiting, pain, diarrhoea, and/or gastric ulcer.[5] It is noteworthy that non-NBPs are unlikely to be associated with such injuries to the jawbones or gastrointestinal tissues.[3,6–9] Indeed, no clear evidence has been found that non-NBPs (etidronate and clodronate) cause such inflammatory or necrotic side effects,[10] despite there having been longer for any such effect of NBPs to become evident (e.g., etidronate was approved in 1977 in USA and in 1990 in Japan). Bisphosphonates (BPs), irrespective of whether they are NBPs or non-NBPs, bind strongly to bone hydroxyapatite, and they accumulate in large amounts upon repeated administration. This accumulation, which is most pronounced in bones exhibiting inflammation, is clearly revealed by bone-scintigraphy in both humans and mice.[11,12] We hypothesized that NBPs that have accumulated in jawbones may be released during and/or after injury or destruction of the jawbones (e.g., due to tooth-extraction and/or infection), and that the released NBPs may directly injure the surrounding soft tissues.[12] Indeed, Scheper et al.[13] detected zoledronate in the saliva of patients who had been treated with zoledronate.

NBPs, when injected into mice intraperitoneally, induce various types of inflammation in a number of organs or tissues (liver, spleen, lung, and bone).[14] Further, subcutaneous injection of NBPs into rats induces necrosis at the injection site,[15] while intradermal injection of NBPs into the ear-pinnas of mice induces inflammation and necrosis.[11,16] It has also been reported that NBPs induce apoptosis in human epithelial cells, but that any such effect of clodronate (non-NBP) is marginal.[17,18] Thus, it is a reasonable proposition that *in vivo* experiments like those mentioned above might be useful for the study of the inflammatory and/or necrotic effects of NBPs. Nevertheless, it remains unclear whether NBPs are indeed toxic to cells within the soft tissues that surround jawbones. Recently, Agis et al.[19] reported an *in vitro* toxicity of zoledronate towards human periodontal fibroblasts, although they doubted whether the effect they described could explain the damage to soft tissues or periodontal tissues induced by NBPs.

Breast cancer, prostate cancer, and lung cancer frequently metastasize to bones. BPs have been demonstrated both experimentally and clinically to be effective for the treatment and prevention of those cancers. After PC-3 cells (a human prostate cancer cell-line) had been inoculated into the tibial bone marrow of mice with severe combined immunodeficiency, the growth of the PC-3 cells was reportedly suppressed by zoledronate.[20] Although it is considered that BPs suppress such tumour growth indirectly, via an inhibition of bone resorption,[21] it is also thought that NBPs may act directly on tumour cells to inhibit their growth,[22] on endothelial cells to impair angiogenesis,[23,24] and on tumour-killing γδT cells to activate them.[25]

Based on the background described above, we thought it of interest to compare the toxic effects of NBPs between periodontal cells and other cells. Interestingly, a consistent finding is that non-NBPs (etidronate and clodronate) are not by themselves inflammatory or necrotic in the above *in vivo* experiments, and actually the inflammation and necrosis induced in mice by NBPs can be reduced or prevented by those two non-NBPs.[11,16,26,27] To test the hypothesis outlined above, we designed the present study to examine the *in vitro* effects of zoledronate and etidronate, alone or in combination, on various human periodontal cells and a number of other cell-types with a variety of origins.

2. Materials and methods

2.1. Reagents

Zoledronate (Toronto Research Chemicals Inc., North York, ON, Canada) and etidronate (Wako Pure Chemical Ind. Ltd., Osaka, Japan) were dissolved in sterile saline, with the pH of the solutions being adjusted to 7 with NaOH, if necessary. Experimental protocols are described in the text or in the legend to the figure relating to each experiment.

2.2. Cells and culture conditions

The following cells were used in the present study: human cementoblasts (HCEM), kindly provided by Dr. T. Takata, Hiroshima University, Hiroshima, Japan[28]; human gingival fibroblasts (HGF), prepared as described previously[29]; human gingival mesenchymal stem cells (HGMS), established as described below; human periodontal ligament cells (HPDL), kindly provided by Dr. E. Nemoto, Tohoku University, Sendai, Japan[30]; human umbilical vein endothelial cells (HUVEC), obtained from Lonza Walkersville Inc., Walkersville, MD, USA; HSC-2 (human squamous carcinoma cell-line), obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University; HSG (human salivary gland adenocarcinoma cell-line), kindly provided by Dr. I. Saito, Tsurumi University, Yokohama, Japan; HSY (human salivary gland adenocarcinoma cell-line), kindly provided by Dr. M. Sato, Tokushima University, Tokushima, Japan; PC-3 (human prostate adenocarcinoma cell-line), obtained from ATCC, Rockville, MD, USA; THP-1 (human monocytic leukaemia cell-line), obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University.

For the establishment of HGMS, single colonies of rapidly growing HGF were passaged. The multipotency of the selected cells was determined by means of a Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems Inc., Minneapolis, MN, USA), used according to the manufacturer's instructions, and cells that displayed multipotency were accepted as HGMS.