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ScienceDirect
British Journal of Oral and Maxillofacial Surgery xxx (2016) xxx–xxx
BRITISH Journal of Oral and Maxillofacial Surgery
www.bjoms.com

Influence of zoledronic acid on proliferation, migration, and apoptosis of vascular endothelial cells

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Accepted 29 May 2016

Abstract

Bisphosphonates are commonly used to treat malignant tumours that originate in bone. In recent years, with the widespread application of these drugs in chemotherapy, reports have raised concerns about the development of osteonecrosis. Though the mechanism of bisphosphonate-related osteonecrosis of the jaw is not clear, vascular injury resulting from their use has been suggested as a possible cause. This study was designed to explore the influence of zoledronic acid, one of the most common bisphosphonates, on the function and viability of vascular endothelial cells in vitro to try to find possible mechanisms of the action bisphosphonates in osteonecrosis. Endothelial cell proliferation, migration, and apoptosis were examined with MTT assays, scratch tests, and flow cytometry in the presence of zoledronic acid in different concentrations. The relative expressions of protein were also examined by western blot assays, and the significance of the differences among concentrations of the drug were assessed statistically.Concentrations zoledronic acid of 15 μmol-150 μmol, can dose-dependently inhibit the activity of vascular endothelial cell proliferation and migration, and significantly up-regulate cell apoptosis ($p < 0.05$). Zoledronic acid can inhibit the vascular endothelial cell activities of proliferation and migration, and can up-regulate cellular apoptosis, which suggests that direct inhibition of angiogenesis together with vascular impairment might contribute to the development of osteonecrosis of the jaw.

Keywords: Bisphosphonate-associated osteonecrosis of the jaw; vascular endothelial cell; proliferation; migration; apoptosis

Introduction

Bisphosphonates have been used in medicine for over 30 years, [1] and their beneficial effect in the treatment of patients with malignant bone neoplasias such as multiple myeloma and bony metastases, or metabolic bone diseases such as Paget's disease and severe osteoporosis, is accepted. However, in recent years, as their use has become more widespread, the number of cases of bisphosphonate-related osteonecrosis of the jaw (BRONJ) has increased, and over two-thirds of them are in the mandible.

The American Association of Oral and Maxillofacial Surgeons has defined BRONJ as exposed bone in the maxillofacial region that has persisted for more than eight weeks, together with current or previous treatment with a bisphosphonate, and no history of radiation to the head and neck.[2] The natural history of osteonecrosis of the jaw generally begins with a superficial mucosal ulcer in either jaw, which

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http://dx.doi.org/10.1016/j.bjoms.2016.05.030

Please cite this article in press as: Lang M, et al. Influence of zoledronic acid on proliferation, migration, and apoptosis of vascular endothelial cells. *Br J Oral Maxillofac Surg* (2016), http://dx.doi.org/10.1016/j.bjoms.2016.05.030

progresses to detectable exposure of bone, and extension of the ulcerated area in breadth and depth with bony necrosis and sequestration. In some serious cases exposed bone is accompanied by pain, infection, and one or more of the following: pathological fracture, extraoral fistula, or osteolysis that extends to the inferior border or sinus floor.[3]

Since 2003, BRONJ has increasingly become the focus of clinical and preclinical investigations, but the mechanism is still not clear. Vascular injury is possible. Studies have shown that zoledronic acid induced a 50% reduction of angiogenesis in the prostate gland in rats, [4] but after three years of daily bisphosphonates given orally to beagles, the necrotic regions showed no patent canaliculi but had retained their vasculature.[5]

In patients with cancer, a single infusion of zoledronate 4 mg induced considerable and long-lasting modifications to circulating angiogenic factors such as vascular endothelial growth factor and platelet-derived growth factor, [6] but histological examination of samples obtained from patients diagnosed with BRONJ showed patent vessels in most cases.[7] The effect of antiangiogenesis and impaired vascularity of bisphosphonates is not therefore delineated at present, and more research is necessary. To understand the mechanisms more clearly we investigated the effect of zoledronate on vascular endothelial cells in vitro.

Material and Methods

Cell culture

Cell cultures were prepared and maintained according to standard procedures. Human umbilical vein endothelial cells line ECV-304 (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle's Medium(Gibco®) supplemented with 1% penicillin–streptomycin–neomycin antibiotic mixture (Gibco®), and 10% fetal calf serum (Gibco®).

Test of cell viability by MTT assays

The effect of zoledronate on the viability of human umbilical vein endothelial cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Solarbio, Science and Technology Company, Beijing). Cells were cultured in 96-well plates (roughly 5×10^3/well) and incubated in growth medium for 24 hours. The cells were treated with different concentrations (varied from 0-500 μmol) of zoledronate for 24 hours, and then incubated for four hours in a medium containing MTT solution 25 μl dissolved in phosphate-buffered saline (PBS, Gibco®) 5 mg/ml, and sterilised. The reaction was terminated by removing medium before adding dimethyl sulphoxide (Vetec) 150 μl and incubating for 10 minutes at 37 °C. The absorbance was measured at 490 nm in an enzyme-linked immunosorbent assay reader (ELISA, SpectraMax®) to assess the viability of the cells. The experiments were repeated at least three times.

Assessment of migration by scratch test

Horizontal migration was assessed with a scratch test. The scratch was made at the bottom of 6-well plates with a small tip along the ruler. The scratch area was washed repeatedly with PBS until the cells had been completely removed. The growth medium with high (150 μmol), medium (50 μmol), and low concentrations (15 μmol) and no zoledronate was added and cultured for 24 hours. Five different fields were selected under an inverted microscope, and the distance between cells at 0 and 24 hours was measured after the scratch while calculating the mean cell migration rate. The cell migration rate = (0 hours scratch width – 24 hours scratch width)/0 hours scratch width × 100%).

Detection of cell apoptosis by flow cytometry

Human umbilical vein endothelial cells were cultured in 6-well plates for 24 hours. Zoledronate was added at various concentrations (0, 15, 50, and 150 μmol), and cultured for another 24 and 48 hours, after which the adherent and non-adherent cells were collected for analysis. To detect apoptosis, harvested cells were double-stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (Becton Dickinson, Mountain View, CA) and propidium iodide (Becton Dickinson) for 5 minutes at room temperature. The populations of annexinV (-)/propidium iodide (-) viable cells and annexinV (+) apoptotic cells were evaluated by flow cytometry (Becton Dickinson). Data were collected in the FACSCalibur™ (Becton Dickinson) and analysed with CellQuest™ software (Becton Dickinson).

Detection of cell cycle by flow cytometry

Human umbilical vein endothelial cells were cultured in 6-well plates for 24 hours, and then incubated with various concentrations of zoledronate (0, 15, 50, and 150 μmol) for 12 hours and harvested. The cells were fixed with 70% ethanol at -20 °C and were stained with 0.5 ml propidium iodide/RNase staining buffer (Becton Dickinson) for 15 minutes at room temperature and analysed by flow cyometry (Becton Dickinson).

Analysis of cell proliferation, migration, and apoptosis by western blotting

Human umbilical vein endothelial cells were cultured in three 6-well plates for 24 hours. Zoledronate was added at various concentrations (0, 15, 50, and 150 μmol) to every plate. Three additional 6-well plates were cultured for another 6, 12, and 24 hours respectively. The protein contents of the cell lysates were measured using the ELISA reader. Proteins were added to 12% sodium dodecyl

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