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Research paper

Intra-tumor delivery of zoledronate mitigates metastasis-induced osteolysis superior to systemic administration

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ABSTRACT

Bisphosphonates (BPs) have recently been shown to have direct anti-tumor properties. Systemic treatment with BPs can have multiple adverse effects such as osteonecrosis of the jaw and BP induced bone fracturing and spine instability. While benefits of systemic BP treatments may outweigh risks, local treatment with BPs has been explored as an alternate strategy to reduce unwarranted risk. In the present study, we examined whether local delivery of BPs inhibits tumor-induced osteolysis and tumor growth more effectively than systemic treatment in an animal model of tumor-induced bone disease. Following establishment of an intra-tibial model of bone metastases in athymic mice, the experimental group was treated by local administration of zoledronate into the tibial lesion. A comparison of the effect of local versus systemic delivery of zoledronate on the formation of tumor-induced osteolysis was also carried out. A significant increase in mean bone volume/tissue volume % (BV/TV) of the locally treated group (12.30 ± 2.80%) compared to the control group (7.13 ± 1.22%) ($P < 0.001$). Additionally, there was a significant increase in the BV/TV (10.90 ± 1.25%) in the locally treated group compared to the systemically treated group (7.53 ± 0.75%) (P=0.005). These preliminary results suggest that local delivery of BPs outperforms both systemic and control treatments to inhibit tumor-induced osteolysis.

1. Introduction

Bone metastases are the most common bone tumor, and they are often derived from solid tumours of the breast, prostate, lung and bladder [1]. Bone metastases are also the most common cause of cancer-related bone pain and often lead to additional complications such as pathological fracture and spine compressions, all of which can severely diminish patients' quality of life [2]. Treatment of bone metastases imposes a huge burden on the healthcare system, and with the advancement in healthcare and increase in cancer life expectancy, metastatic bone disease is projected to increase dramatically [3]. Current treatment options for bone metastases are surgical therapy, radiotherapy, anti-receptor activator of nuclear factor-kappaB ligand (RANKL) antibody, and systemic bisphosphonates (BPs).

During bone turnover, osteoblasts build bone while osteoclasts are responsible for bone removal [4]. BPs are potent anti-resorptive agents that reduce bone resorption by inhibiting osteoclastic cell activity. Many studies have shown that there is an increase in osteoclast numbers and activity in metastatic bone disease. As such, the anti-resorptive activity of BPs has been explored to reduce bone cancer pain, bone destruction, and bone tumor growth [5]. Interestingly, BPs have also been suggested to have anti-tumor properties by negatively regulating macrophages, endothelial cells and tumor cells [4,6]. BPs have also been shown to elicit combinatorial effects with chemotherapeutic agents. They are often administered to breast or prostate cancer patients with metastatic bone disease as a single intravenous dose or course of treatment as part of the standard care regimen [7,8]. Local delivery of BPs by elution from porous materials can be used to enhance bone formation, suggesting a different approach to systemic treatment [9].

Due to close proximity to vital structures, incomplete surgical resection of bone metastases is common. The remaining tumor can promote osteolysis of the surrounding bone. While systemic BP treatment is often given to these patients, several complications such

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as osteonecrosis of the jaw, atrial fibrillations, hypocalcemia and acute inflammatory response may render systemic BPs administration unsuitable for some patients [10]. Therefore, we sought to examine whether local delivery of BPs can inhibit tumor-induced osteolysis and tumor growth in an animal model of tumor-induced bone disease. We also sought to determine whether the efficacy of local delivery of BPs is comparable to that of systemic BPs on disrupting tumor-induced osteolysis.

2. Materials and methods

2.1. Study subjects and participants

MDA-MB-231/LUC cell line (Cedarlane, ON, Canada) were cultured in a DMEM cell culture medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotics (HyClone brand from Thermo Scientific) at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO2). Zoledronic acid was purchased from Sigma, USA, and D-luciferin was purchased from PerkinElmer, USA. The experimental design used 35 female athymic nude mice (490, Homozygous), aged 9–12 weeks, purchased from Charles River, USA. The average weight was 25 g (range, 22.7–27.6 g). The mice were maintained in pathogen-free conditions. The Mcgill Animal Care and Use Committee approved all the experimental procedures.

2.2. Establishment of an intra-tibial mice model of bone metastasis

MDA-MB-231/LUC cell line (N=10^5) were resuspended in 20 μl of Phosphate buffered saline (PBS) and injected into the marrow space of the right tibia using a 27½ gauge needle coupled to a Hamilton syringe under imaging guidance [11]. Five days following inoculation, the presence of tumor cells was confirmed using in vivo bioluminescence imaging (IVIS spectrum, PerkinElmer, USA). Mice were randomly assigned to different groups according to the design of each experiment. At the end of each experiment, the mice were sacrificed using the American Association for Laboratory Animal Science (IACUC) approved method (CO2 asphyxiation).

2.3. Intra-tibial administration of zoledronate

Mice were anesthetised using gas anesthetics and were placed in supine position over a heated pad to avoid hypothermia. The site of the injection was draped in a sterile fashion and cleaned by 70% ethanol swab. The ipsilateral knee was flexed to 90 degrees. Following this a 27½ gauge (½ inch long) needle attached to a 1 ml syringe was inserted percutaneously through the knee joint. The syringe axis was kept in alignment to the axis of the tibia. The advancement of the needle was carried on under image guidance to make sure that we are in the metaphyseal bone of the proximal tibia. Proximal tibia was drilled by rotating the syringe half to ¾ run. Then, another syringe loaded with zoledronate was inserted at the tract made by drilling. After successful administration of zoledronate, animals were monitored till complete recovery from anesthesia. Systemic analgesia was administered for 48–72 h following the procedure.

After successful inoculation of MDA-MB-231/LUC cell line, mice were divided randomly into two separate groups, the treatment group (2 μg of zoledronate suspended in PBS (30 μl) /mice, delivered intra-tibially three times/week for three weeks, N =11), and the control group (PBS (30 μl) /mice, intra-tibially three times/week for three weeks, N=5), as described above. Zoledronate treatment was started one week after the successful implantation of tumor cells. The tumor growth was monitored weekly using *in vivo* bioluminescence imaging and clinically for any signs of tumor development. After three weeks of treatment, the mice were sacrificed and tibias were removed and dissected for micro-computed tomography (Skyscan1172, Skyscan, Belgium) and histological analysis.

2.4. Local versus systemic administration

Following successful injection of MDA-MB-231/LUC cell line, nineteen athymic nude mice were divided randomly into two groups: the local treatment group (0.025 mg/kg of zoledronate in PBS (30 μl), delivered intra-tibially once/week for four weeks, N=6), the systemic treatment group (0.025 mg/kg zoledronate in PBS (100 μl), delivered sub-cutaneously once/week for four weeks, N=5). Doses were calculated based on an average weight of 25 g. [12] The treatment was started one week following successful inoculation of the breast cancer cells. After four weeks of treatment, the mice were sacrificed, and tibias were removed and dissected for micro-computed tomography and histological analysis.

2.5. In vivo bioluminescence imaging

The growth of MDA-MB-231 derived tibial lesions was assessed by longitudinal bioluminescence imaging. The mice were imaged using IVIS spectrum following an intra-peritoneal injection of D-luciferin solution (PerkinElmer, USA) (150 mg/kg body weight) under gas anesthetic. Bioluminescence images were taken 20 min after D-luciferin injection and acquired until the peak signal was reached. Photon emission was quantified using Living Image software and graphed according to the average radiance (photons/s/cm^2/sr).

2.6. Micro-computed tomography (μ -CT) analysis

Tibiae were dissected from mice at necropsy and excised tibia scanned using a high-resolution micro-tomographic system. Each of the three-dimensional images was constructed from approximately 550 individual micro-CT images (8.9 μm/image) starting from the growth plate of the tibia and moving distally. Image reconstruction was performed using NRecon (Version 1.6.2.0; SkyScan). The CT analyzer (1.11.8.0; SkyScan) was used to measure static histomorphometric parameters of the region of interest including bone volume/ tissue volume % (BV/TV), trabecular number, trabecular thickness and trabecular separation.

2.7. Histology and immunohistochemistry

Histological analysis of tumor burden was performed on hematoxylin and eosin (H & E) stained sections of tibias. Imagescope software (Aperio) was used to quantify tumor area in mm^2. Tumor burden was expressed as the proportion of tumor occupying the total area. Tissue fixation and immunohistochemical (IHC) staining were carried out as previously described. [13] The proliferative index in the bone metastatic lesions was assessed by staining with a Ki67 antibody (1 μg/ml; Abcam, Toronto, ON, Canada). Anti- Cleaved-Caspase 3 staining (0.2 μg/ml dilution; Cell Signaling, Whitby, ON, Canada) was performed to quantify apoptosis within the bone lesions. Following incubation with the primary antibody, secondary biotin-conjugated antibody (Jackson Laboratories) was applied for 30 min. After washing with distilled water, slides were developed with diaminobenzidine (Dako) as the chromogen. All slides were counterstained using Harris haematoxylin before being scanned using a Scanscope XT digital slide scanner (Aperio). Quantification was performed by analyzing bone metastases with Imagescope software (Aperio) using positive pixel count algorithm for Ki67 and Cleaved Caspase-3 staining. For quantification of Ki67 and Cleaved Caspase-3 staining, positively stained nuclei were represented as a percentage of total nuclei per field.

2.8. Statistical analysis

All statistical analyses were conducted using SPSS Version 21 (Armonk, NY: IBM Corp). All data were expressed as Mean ± SEM. Non-parametric two-sided Mann-Whitney *U*-tests was applied to test

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