Bone 66 (2014) 240–250

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Modifying the osteoblastic niche with zoledronic acid *in vivo*—Potential implications for breast cancer bone metastasis

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ARTICLE INFO

Article history:
Received 21 March 2014
Revised 12 June 2014
Accepted 18 June 2014
Available online 24 June 2014

Edited by: J. Aubin

Keywords:
Metastatic niche
Osteoblast
Osteoclast
Breast cancer
Homing

ABSTRACT

Introduction: Bone metastasis is the most common complication of advanced breast cancer. The associated cancer-induced bone disease is treated with bone-sparing agents like zoledronic acid. Clinical trials have shown that zoledronic acid also reduces breast cancer recurrence in bone; potentially by modifying the bone microenvironment surrounding disseminated tumour cells. We have characterised the early effects of zoledronic acid on key cell types of the metastatic niche *in vivo*, and investigated how these modify the location of breast tumour cells homing to bone.

Methods: Female mice were treated with a single, clinically achievable dose of zoledronic acid (100 μg/kg) or PBS. Bone integrity, osteoclast and osteoblast activity and number/mm trabecular bone on 1, 3, 5 and 10 days after treatment were assessed using μCT, ELISA (TRAP, PINP) and bone histomorphometry, respectively. The effect of zoledronic acid on osteoblasts was validated in genetically engineered mice with GFP-positive osteoblastic cells. The effects on growth plate cartilage were visualised by toluidine blue staining. For tumour studies, mice were injected i.c. with DID-labelled MDA-MB-231-NW1-luc2 breast cancer cells 5 days after zoledronic acid treatment, followed by assessment of tumour cell homing to bone and soft tissues by multiphoton microscopy, flow cytometry and *ex vivo* cultures.

Results: As early as 3 days after treatment, animals receiving zoledronic acid had significantly increased trabecular bone volume vs. control. This rapid bone effect was reflected in a significant reduction in osteoclast and osteoblast number/mm trabecular bone and reduced bone marker serum levels (day 3–5). These results were confirmed in mice expressing GFP in osteoblastic linage cells. Pre-treatment with zoledronic acid caused accumulation of an extra-cellular matrix in the growth plate associated with a trend towards preferential [1] homing of tumour cells to osteoblast-rich areas of bone, but without affecting the total number of tumour cells. The number of circulating tumour cells was reduced in ZOL treated animals.

Conclusion: A single dose of zoledronic acid caused significant changes in the bone area suggested to contain the metastatic niche. Tumour cells arriving in this modified bone microenvironment appeared to preferentially locate to osteoblast-rich areas, supporting that osteoblasts may be key components of the bone metastasis niche and therefore a potential therapeutic target in breast cancer.

Introduction

The majority of cancer deaths are due to metastatic disease, and the lack of effective anti-metastatic therapies reflects our incomplete understanding of the underlying biology of tumour cell spread. Breast, prostate and lung cancers are amongst the most common malignancies with a preference to metastasise to the skeleton [1]. Treatment at this stage is palliative and often includes a bone-sparing anti-resorptive bisphosphonate (BP) [2], with zoledronic acid being the most potent [3]. In breast cancer, the dissemination of malignant cells to bone is thought to be an early event and tumour cells may reside in a dormant state within the bone for many years before developing into the

Abbreviations: BP, Bisphosphonate; ECM, Extracellular matrix; GFP, Green fluorescent protein; HSC, Hematopoietic stem cell; NBP, Nitrogen-containing bisphosphonate; PBS, Phosphate buffered saline; ROI, Region of interest; TRAP, Tartrate resistant alkaline phosphatase; PINP, Procollagen type 1N-terminal propeptide; ZOL, Zoledronic acid.

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http://dx.doi.org/10.1016/j.bone.2014.06.023

incurable secondary disease [4]. Elucidating the signals that maintain tumour cell dormancy, as well as the triggers for escape to a proliferative state, is currently one of the most intensely studied areas of cancer biology.

There is a general consensus that components of the bone marrow microenvironment make up a 'bone metastasis niche', responsible for regulating tumour cell homing, survival and dormancy. To what extent this overlaps with the hematopoietic stem cell niche, a specialised microenvironment that regulates hematopoietic stem cell (HSC) function, survival and quiescence, is not fully established [5–7]. The HSC niche is described to include an endosteal niche, of which the main cellular components are cells of the osteoblastic lineage [8]. In addition to regulating HSCs, it is proposed that the same niche may be creating a beneficial microenvironment for disseminated tumour cells in bone. Using *in vivo* model systems, Shiozawa *et al.* have shown that prostate cancer cells and HSCs reside within the same niche in the bone marrow [7] and that disseminated tumour cells can displace HSCs from the niche resulting in growth of metastatic colonies [9,10]. This suggests that components of the HSC niche, including osteoblastic cells, may be involved in tumour cell homing to bone. However, it remains to be established whether the osteoblast is a critical component of the metastatic niche, as well as the specific role of the tightly coupled osteoclast. In a breast cancer xenograft model, we have demonstrated that both osteoblast and osteoclast number/mm trabecular bone surface is significantly altered by breast tumour colonies at early and advanced stages of bone metastasis, indicating that both cell types may be intimately linked to tumour progression [11].

Therapeutic targeting of the bone microenvironment with anti-resorptive agents is standard of care for breast cancer patients with established cancer-induced bone disease [2]. Intriguing data from the AZURE trial demonstrated increased survival and reduced bone metastases when zoledronic acid is given in the adjuvant setting [12]. Several *in vivo* studies have also reported that inhibiting osteoclastic bone resorption with BPs early in the development of bone metastases reduces cancer-induced bone disease and may slow down disease progression. Preventive treatment with BPs (prior to tumour cell injection) is shown to be more effective at reducing tumour growth in bone when compared to therapeutic scheduling (initiated once bone metastases are established). For example, ibandronate treatment (10 μg/kg/day) of animals with established intrafemoral MDA-MB-231 tumours reduced progression of osteolytic lesions and metastases but did not eliminate tumour growth and larger lesions were unaffected [13]. In contrast, when treatment was initiated prior to cancer cell injection (day −3), formation of new osteolytic lesions and incidence of metastases were reduced. Alterations to the metastatic site before tumour cell arrival may thus impede tumour cell engraftment in bone, which could result in more pronounced anti-tumour effects. Another study suggesting that the reported anti-tumour effects of bisphosphonates are due to alterations of the bone microenvironment showed that preventive treatment with olpadronate (1.6 μmol/kg/day, 2 days before MDA-MB-231 tumour cell injection) significantly reduced new bone metastasis formation, while a therapeutic protocol (1.6 μmol/kg/day, day 28 to day 46) did not affect tumour growth in bone [14]. The authors suggest that the preventive schedule reduced tumour growth by inhibiting the release of tumour growth factors by osteoclastic bone resorption, an established mechanism for driving progression of cancer-induced bone disease.

Most studies investigating BP-induced anti-tumour effects did not investigate the consequences of inhibiting osteoclast activity on the tightly coupled osteoblasts. As both cell types are now suggested to be part of the metastatic niche, it is of great interest to determine how preventive scheduling of anti-resorptive agents modify osteoblasts, and the potential implications for subsequent tumour cell homing and colonisation. It is possible that development of bone metastases is also inhibited by BPs modifying the size and/or availability of the metastatic (osteoblastic) niche. The available data on the potential direct vs. indirect effects of bisphosphonates on osteoblasts is somewhat contradictory, with studies reporting a reduction of osteoblast activity and survival *in vitro* and *in vivo* [15–18] while others have reported a beneficial effect of NBP treatment on osteoblast development, survival and growth [19–21].

We hypothesise that modification of the cellular components of the bone metastasis niche by zoledronic acid may affect the ability of tumour cells to initiate bone metastasis. Here we present the first *in vivo* study to assess the early (days 1–10) effects of a single, clinically relevant, dose of zoledronic acid on the bone microenvironment suggested to be part of the bone metastatic niche, osteoblasts and osteoclasts. In addition, we determined how these rapid, zoledronic acid-induced, changes to the bone microenvironment may affect early colonisation of particular areas of bone by breast cancer cells.

Materials and methods

Animal models and drug treatment

BALB/cAnNCrl *Foxn1*$^{nu/nu}$ immunocompromised (athymic nude) mice were obtained from Charles River (Kent, UK). A transgene engineered to express GFP under the control of type 1 collagen promoter (pOBCol2.3GFPemd, kindly provided by Prof. David Rowe, University of Connecticut, USA) was introduced into the BALB/cAnNCrl nude mice by repeated backcrossing to generation N_5. Heterozygous nude mice were then intercrossed to generate homozygotes. This line (BALB/cAnNCrl.Cg-Tg(Col1a1-GFP)Row *Foxn1*$^{nu/nu}$) results in immunocompromised mice expressing GFPemd in cells of the osteoblast lineage and was used as a model to investigate the link between disseminated cancer cells and resident osteoblasts. Mice were housed in a controlled environment with a 12 h light/dark cycle at 22 °C. They were provided with *ad libitum* access *2018 Teklad* Global 18% protein rodent diet containing 1.01% Calcium (Harlan Laboratories, UK) in individually ventilated cages (Tecniplast, Milan, Italy). All *in vivo* experiments complied with the UK Animals (Scientific Procedures) Act 1986 and were reviewed and approved by the local Research Ethics Committee of the University of Sheffield (Sheffield, UK). All work was performed under Home Office regulations (project licenses 40/3462 and 40/3531).

Cohorts of 6- and 12-week old female immunocompetent BALB/c or 6-week old immunocompromised BALB/c nude mice ($n = 2$–7/group) were used to assess the effect of zoledronic acid treatment on bone cells. To investigate the effects on osteoblasts in greater detail 6- and 10-week-old transgenic male mice ($n = 2$/group) expressing GFP-positive osteoblastic cells on a BALB/c nude background (described above) were used.

Animals were randomised into two treatment groups: (1) PBS and (2) zoledronic acid (ZOL, 100 μg/kg i.p.; supplied as disodium salt by Novartis; equivalent to the 4 mg infusion used in the treatment of cancer-induced bone disease) and sacrificed on days 0, 1, 3, 5 and 10 post injection (Fig. 1A). Hind legs were collected and fixed in either 10% buffered formalin prior to μCT analysis or 4% PFA solution before decalcification (0.5 M EDTA, 0.5% PFA, PBS, pH 8). Blood was collected and spun down at 4000 rpm for 10 min at 4 °C and serum was stored at −80 °C prior to analysis of serum bone turnover markers.

Bone metastasis model

To assess the effect of pre-treatment with ZOL on tumour cell homing to bone 8–13-week old female BALB/c mice (heterozygote or homozygote nude) with GFP expressing cells of the osteoblast linage were injected with PBS or ZOL (100 μg/kg i.p.) on day 0. Animals were injected with 1×10^5 MDA-MB-231-NW1 (luc2 positive) breast cancer cells intracardiac and sacrificed on day ten (Fig. 1B). Immediately before injection, tumour cells were labelled with the lipophilic dye DID (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Whole blood was collected, all samples from one treatment group were pooled, red blood cells were lysed, and the number of DID positive (DID+) tumour cells/mL was determined by flow cytometry. Bone