Bone 50 (2012) 723–732

Contents lists available at SciVerse ScienceDirect

Bone

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

Original Full Length Article

Krüppel-like factors KLF2 and 6 and Ki-67 are direct targets of zoledronic acid in MCF-7 cells

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ARTICLE INFO

Article history:
Received 30 March 2011
Revised 25 November 2011
Accepted 26 November 2011
Available online 7 December 2011

Edited by: J. Aubin

Keywords:
MCF-7
Zoledronic acid
Mevalonate pathway
Krüppel-like factors
Ki-67

ABSTRACT

Bisphosphonates (BP) are used for the treatment of osteoporosis and bone metastases due to breast and prostate cancer. Recent clinical studies indicated a benefit in survival and tumor relapse with the supportive treatment of breast cancer using zoledronic acid (ZA), thus stimulating the debate about its putative anti-tumor activity in vivo. MCF-7 breast cancer cells were treated for 3 h (pulse treatment) and 72 h (permanent treatment) with ZA, and apoptosis rates and cell viability, defined as ATP content, were determined after 72 h. Permanent and pulse stimulation with ZA inhibited the viability of MCF-7 cells, which could partly be rescued by atorvastatin (Ator) pre-treatment but not by geranylgeranyl pyrophosphate (GGPP) co-treatment. Microarray analysis of ZA treated MCF-7 cells identified genes of the mevalonate pathway as significantly upregulated, which was verified by qPCR. Additionally the putative tumor suppressors krüppel-like factor 2 and 6 (*KLF2* and *KLF6*) were markedly upregulated, while the classical proliferation marker *Ki-67* was clearly downregulated. The expression of all three genes was confirmed by qPCR on mRNA level and by immunocytochemistry or Western blot staining. Expression of target genes were also analyzed in other breast (MDA-MB-231, BT-20, ZR75-1, T47D) and prostate (LNCaP, PC3) cancer cell lines by qPCR. ZA responsiveness of *KLF2*, *KLF6* and *Ki-67* could be verified in PC3 and T47D cells, *KLF6* responsiveness in LNCaP and *KLF2* responsiveness in MDA-MB-231 and BT-20 cells. Here we demonstrate in the apoptosis insensitive MCF-7 cell line a remarkable impact of ZA exposure on cell viability and on the regulation of putative tumor suppressors of the KLF family. The molecular mechanism involved might be the accumulation of isopentenyl pyrophosphate (IPP) and ApppI, since we could partly rescue the ZA effect by Ator pre-treatment and GGPP co-treatment. These data should stimulate further research into both the role of the mevalonate pathway and the accumulation of pyrophosphate compounds like ApppI in tumorigenesis and differentiation and their potential apart from the inhibition of mitochondrial ADP/ATP translocase and apoptosis, since such effects might well be responsible for the adjuvant ZA treatment benefit of patients suffering from breast cancer.

Abbreviations: AACS, AcylCoA synthetase short chain family member 2; ACAT2, AcetylCoA acetyl transferase; ACSS2, acetoacetyl-CoA synthetase; ApppI, triphosphoric acid 1-adenosin-50-yl ester 3-(3-methylbut-3-enyl) ester; Ator, atorvastatin; BPs, bisphosphonates; CHM, choroideremia (Rab escort protein 1); EMT, epithelial mesenchymal transition; ER, estrogen receptor; FCS, fetal calf serum; FDPS, farnesyl pyrophosphate synthase; FDFT1, farnesyldiphosphate farnesyltransferase; FPP, farnesyl pyrophosphate; FNTA, farnesyltransferase alpha; FNTB, farnesyltransferase beta; FTI, farnesyltransferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGPS1, geranylgeranyl pyrophosphate synthase; GGTI, geranylgeranyl transferase inhibitor; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl CoA synthase; hMSC, human mesenchymal stem cells; IDI1, isopentenyl pyrophosphate isomerase; KLF, krüppel like factor; LLS, lanosterol synthase; MET, mesenchymal epithelial transition; MVD, mevalonate pyrophosphate decarboxylase; MVK, mevalonate kinase; PBS, phosphate buffered saline; PGGT1B, protein geranylgeranyl transferase type I, beta subunit; PMVK, phosphomevalonate kinase, qPCR, quantitative polymerase chain reaction; RABGGTA, Rab geranylgeranyltransferase, alpha subunit; RABGGTB, Rab geranylgeranyl transferase, beta subunit; SQLE, squalene epoxidase; ZA, zoledronic acid.

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8756-3282/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.bone.2011.11.025

Introduction

Bone metastases occur in many tumors but exceptionally often in cancers derived from the mammary gland, the prostate and the kidney. The burden of metastatic bone disease is high, caused by bone pain and pathologic fractures, which initiate hospitalization, radiation and operative intervention. The biological process of metastases requires a series of special features of tumor cells, e.g. the capability of extravasation, migration and homing to the bone microenvironment. The underlying process for metastases in epithelial cancers is regularly accompanied by epithelial mesenchymal transition (EMT), a process which is basically reversible (MET) but may also be propagated to an extent that the similarity achieved between tumor cells and mesenchymal cells has been coined "osteomimicry" [1–3]. The advantages of tumor cells in acquiring mesenchymal attitudes are clearly adhesion and homing advantages as well as the utilization of bone microenvironment, e.g. the activation of osteoclasts by secreting osteoclast promoting factors. Tumor cells create space by stimulating osteoclasts, which also releases latent growth factors buried in bone [4]. These molecular mechanisms are the basis of the classical concepts of osteolytic and osteoblastic metastases.

Bisphosphonates (BPs) show a high affinity to hydroxylapatite and their accumulation in bone results in osteoclast inhibition and apoptosis [5]. The molecular mechanism of action of first generation BP like clodronate is the induction of apoptosis by accumulating toxic ATP adducts. Second generation Amino-BP very specifically inhibit farnesyl pyrophosphate synthase, an enzyme of the mevalonate pathway leading to an inhibition of protein prenylation of e.g. small GTP binding proteins like Rab, Ras, RhoA or lamins, which are important for cellular polarization and cytoskeleton organization [6,7]. Additionally it was reported for zoledronic acid (ZA) that treatment of cells produced a new endogenous ATP analog (triphosphoric acid 1-adenosin-50-yl ester 3-(3-methylbut-3-enyl) ester, abbreviated to 'ApppI'), which inhibited the mitochondrial ADP/ATP translocase and caused apoptosis in osteoclasts [8].

When applied in higher doses than used for osteoporosis, BPs effectively reduced bone pain and the number of skeletal related events in patients with bone metastases [9,10]. These effects and proven clinical efficacy has made them to an important class of drugs in the treatment of osteolytic bone diseases during the last two decades [11]. Besides the effects on their classical targets, cells of the myelomonocytic/macrophage lineage and especially osteoclasts, BPs have been shown to induce apoptosis in a variety of benign and malignant cells, although in some cases μM concentrations were required [5]. These in vitro effects stimulated discussions about a putative antitumor effect of BP. Almost twenty years ago it was first shown that adjuvant treatment with BPs reduces the incidence of bone and soft tissue metastases as well as the overall mortality in patients suffering from breast cancer. These results have recently been confirmed in the ABCSG-12 trial, where ZA was used only twice a year for the adjuvant treatment of estrogen receptor (ER) positive patients. But also long lasting effects in the first cohort were reported in a second analysis more than ten years after the first publication [12–14]. Moreover, a synergistic anticancer efficacy of ZA in combination with neoadjuvant chemotherapy was shown in breast cancer patients with respect to additional tumor shrinkage [15].

The detailed characterization of the molecular effects of modern BPs like ZA stimulated research about their effects on both osteoblastic differentiation and on anti tumor effects. We and others have previously demonstrated sustained effects on osteogenic differentiation upon both low dose treatment and intermittent high dose treatment with e.g. ZA and alendronate [16,17], while permanent exposure to high concentrations of such BPs may induce apoptosis in both osteogenic precursors and tumor cells [16,18,19]. Analyzing MDA-MB-231 ER negative breast cancer cells and ER positive MCF-7 cells we have demonstrated that ZA is able to modulate the osteoprotegerin/TRAIL gene expression ratio in MDA-MB-231 cells but not in MCF-7 cells. This is of special concern as MDA-MB-231 cells are sensitive to TRAIL-induced apoptosis [20]. With respect to differential effects of various BPs on tumor cell growth and apoptosis it is of major importance to unravel their differential potency and to describe their downstream targets in non-osteoclastic cells.

Both, the inhibition of apoptosis and the enhanced capacity for self-renewal are characteristics of tumor cells. Krüppel-like factors (KLF) play an important role in endocrine cancers of female reproductive tissues. KLFs are a family of transcription factors consisting of 17 members, which are involved in a series of important biological processes including control of apoptosis, migration and proliferation. They are capable of modulating steroid hormone action and can both act as tumor suppressors or as oncogenes [21]. Especially in breast cancer cells KLFs have been associated with enhanced proliferation and invasiveness. KLF6 promotes cell cycle arrest via interaction with cyclin D1, thereby disrupting the phosphorylation of retinoblastoma protein [22]. KLF2 is required for normal vessel formation and a regulator of endothelial function, overexpression of KLF2 leads to an inhibition of angiogenesis [23]. Another important proliferation marker for breast cancer is Ki-67, which is positively associated with shorter overall survival. In neoadjuvant endocrine studies it was shown that the detection of changes in Ki-67 predicts for breast cancer treatment benefit [24]. It is also under debate if guidelines should be changed to include Ki-67 in the standard pathological assessment of early breast cancers [25].

Here we show that the breast cancer cell line MCF-7 in vitro permanently and pulse treated with ZA show reduced cellular viability, defined as ATP content, which can partly be rescued with statin pretreatment. In MCF-7 cells the expression of genes involved in the mevalonate pathway as well as putative tumor suppressor genes of the krüppel-like family of transcription factors were induced and the expression of the proliferation marker Ki-67 was reduced after 72 h of permanent exposure to ZA, indicating relevant effects on tumor cell biology, which might be a molecular basis of the positive clinical results in the adjuvant setting of breast cancer.

Materials and methods

Cell culture

Media for cell culture and fetal calf serum (FCS) were obtained from PAA Laboratories (Linz, Austria). MCF-7 cells (ER positive) were cultivated in DMEM high glucose supplemented with 1 mM pyruvate and 10 μg/ml insulin (both Sigma Aldrich GmbH, Schnelldorf, Germany), T47D (ER positive) breast cancer cells were cultivated in RPMI1640 medium supplemented with 10 μg/ml insulin. MDA-MB-231, BT-20 (ER negative) and ZR75-1 (ER positive) breast cancer cells were cultivated in DMEM/F12, LNCaP and PC3 prostate cancer cells were cultivated in RPMI1640 medium. All media were supplemented with 10% FCS and penicillin/streptomycin. Primary human mesenchymal stem cells (hMSC) were isolated from bone marrow from different donors and cultivated up to four weeks by a standardized protocol as described previously [16,26]. Bone marrows were recovered after informed consent from the explanted femoral heads of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. All cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Cell viability and apoptosis assays

For determination of long-term effects on cell viability and apoptosis, MCF-7 cells were seeded on 96-well plates with a density of 1000 cells/well and were stimulated with 50 μM zoledronic acid (ZA) for 72 h. To block effects of 50 μM ZA, cells were co-treated for