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Ibandronate increases the expression of the pro-apoptotic gene FAS by epigenetic mechanisms in tumor cells

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ABSTRACT

There is growing evidence that aminobisphosphonates like ibandronate show anticancer activity by an unknown mechanism. Biochemically, they prevent posttranslational isoprenylation of small GTPases, thus inhibiting their activity. In tumor cells, activated RAS-GTPase, the founding member of the gene family, down-regulates the expression of the pro-apoptotic gene *FAS* via epigenetic DNA-methylation by DNMT1. We compared ibandronate treatment in neoplastic human U-2 osteosarcoma and in mouse CCL-51 breast cancer cells as well as in the immortalized non-neoplastic MC3T3-E1 osteoblastic cells. Ibandronate attenuated cell proliferation in all cell lines tested. In the neoplastic cells we found up-regulation of caspases suggesting apoptosis. Further we found stimulation of *FAS*-expression as a result of epigenetic DNA demethylation that was due to down-regulation of DNMT1, which was rescued by re-isoprenylation by both geranylgeranyl-pyrophosphate and farnesylpyrophosphate. In contrast, ibandronate did not affect *FAS* and DNMT1 expression in MC3T3-E1 non-neoplastic cells. Data suggest that bisphosphonates via modulation of the activity of small-GTPases induce apoptosis in neoplastic cells by DNA-CpG-demethylation and stimulation of *FAS*-expression. In conclusion the shown epigenetic mechanism underlying the anti-neoplastic activity of farnesyl-transferase-inhibition, also explains the clinical success of other drugs, which target this pathway.

1. Introduction

The 3rd generation of aminobisphosphonates like alendronate, risedronate, zoledronic acid, ibandronate and other compounds affect bone resorption of osteoclasts by inhibiting isoprenylation of the small GTP-binding proteins and are therefore used as anti-resorptive treatment of osteoporosis. Additionally to their effects on bone, there is growing evidence for an anticancer activity of these drugs [1–8]. However, the mechanisms involved in these effects remain poorly understood.

Biochemically, aminobisphosphonates act principally by inhibiting farnesyl pyrophosphate (FPP) synthase – an enzyme of the mevalonate pathway – thereby preventing the post-translational modification (prenylation) of small guanosine triphosphate (GTP)-binding proteins (small-GTPases) that is essential for their function. Because small GTP-binding proteins modulate nearly every cellular activity, it is clear that functional inhibition from members of this protein family influences growth and differentiation of normal cells as well as of tumor cells [3,5,7,9–11].

The founding members of the large family of small-GTPases are the three RAS-proteins, HRAS, NRAS and KRAS. Activation of the RAS/RAF/MEK/ERK pathway, for instance by mutation of the involved GTPases or their regulating members, is responsible for the development of a plethora of cancers [12–14] and targeting this pathway seems to be a promising strategy in tumor therapy [15,16]. The RAS/RAF/MEK/ERK pathway activates DNA-methylation processes [17–19]. Epigenetic processes include histone modifications and methylation of CpGs (cytosine-guanosine dinucleotides) on the DNA, especially on gene promoters. Changes of the methylation state of gene promoters lead to alteration in gene expression patterns. This influences cellular differentiation and apoptosis and thus tumor formation (for review see Refs. [19–22]). DNA methylation dependent inactivation of tumor suppressor genes like cell cycle inhibitors (e.g. *CDKN1A*, *CDKN1B*, *CDKN2A*, *CDKN2B*) and *LOX* (lysyl oxidase) as well of the pro-apoptotic gene *FAS* (TNF receptor superfamily, member 6) is often observed during development of neoplastic diseases. Promoter CpG-hypermethylation of these genes was found in colon cancers [23], prostate carcinomas [24–26], breast cancers [27–29] or hematologic malignancies [30–33]. Consequently, several DNA demethylating

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agents were developed and are now in use as anti neoplastic drugs to reactivate genes such as FAS which plays a key role in immortality of cancer stem cells [34].

It has recently been shown that activated RAS prevents cellular apoptosis by epigenetic inhibition of *Fas* expression through stimulation of the RAF/MEK/MAPK1 pathway with subsequent *Fas* promoter methylation via DNMT1 (DNA-(cytosine-5-)-methyltransferase 1), an enzyme responsible for CpG methylation during cell replication [17]. Similarly, in osteoblasts, extracellular matrix (collagen type I) preserves CpG-methylation of the *Fas* promoter via MAPK1 and DNMT1, thus preventing apoptosis of proliferating osteoblasts [19]. Although, utmost efforts have been spent to clarify the relevance and the regulation of cytosine methylation for physiological and pathological development, only few progresses have been made until now. The involvement of RAS and other small GTP-binding proteins in bisphosphonates' activity and the knowledge of apoptotic effects on bone cells, also of bisphosphonates of the 3rd generation [1,4,6,7,35–37], suggest that these drugs could modulate CpG-methylation of gene promoters.

Here, we demonstrate that the aminobisphosphonate ibandronate modulates the DNA methylation status of the *FAS* promoter by influencing the isoprenylate pathway in human U-2 osteosarcoma (OS) cells and CCL-51 cells, a murine mammary gland tumor cell line, but not in non-neoplastic immortalized MC3T3-E1 cells. Treatment with ibandronate leads to re-expression of FAS and to increased activity of apoptosis-associated caspases in the tumor cell lines. Knock down of *FAS* mRNA expression by siRNA technique largely re-establishes cell viability in ibandronate treated neoplastic U-2 OS cells. Our data suggest that epigenetic mechanisms play a key role in the apoptotic activity of bisphosphonates, and possibly many of their effects on cellular physiology including systemic changes within an organism.

2. Materials and methods

2.1. Cell culture

MC3T3-E1 cells, a clonal pre-osteoblastic cell line derived from newborn mouse calvaria (kindly donated by Dr. Kumegawa, Meikai University, Department of Oral Anatomy, Sakado, Japan) and the human osteosarcoma cell line U-2 OS were cultured in alpha-minimum essential medium (α-MEM; Biochrom, Berlin, Germany) supplemented with 50 μg/mL ascorbic acid (Sigma–Aldrich, St. Louis, MO), 5% fetal calf serum (Biochrom), and 10 μg/mL gentamycin (Sigma–Aldrich). CCL-51 cells, a murine mammary gland tumor cell line, were cultured in eagle minimum essential medium (EMEM, Sigma–Aldrich) supplemented with 292 μg/mL L-glutamine, 10% fetal calf serum and 10 μg/mL gentamycin. All cells were cultured in humidified air under 5% CO_2 at 37 °C. For propagation, cells were subcultured twice a week using 0.001% pronase E (Roche, Mannheim, Germany) and 0.02% EDTA in Ca2+- and Mg2+-free phosphate-buffered saline (PBS) before achieving confluence. To prevent a potential phenotypic drift during repeated sub-cultures, the cells were not used for more than 4 weeks after thawing. For experiments, cells were seeded in culture dishes at a density of 20,000/cm^2 as untreated controls or treated with the indicated compounds at times and concentrations specified. Ibandronate, geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP) were purchased from Sigma-Aldrich. Ibandronate was dissolved in water and aliquots were frozen at −20 °C.

2.2. Cell viability/proliferation

To assess cell metabolic activity, a commercially available, MTT similar assay (EZ4U; Biomedica, Vienna, Austria) was used. For this purpose, the cell lines were incubated with increasing concentrations of ibandronate (1–50 μM for MC3T3-E1 and U-2 OS cells or 1–200 μM for CCL-51 cells). After a comparable doubling time for all three cell lines the assay was performed following the protocol of the supplier.

2.3. Cell counting

Cells were seeded in 24 multi-well culture dishes at a density of 20,000/cm^2 and were either left untreated (controls) or treated with ibandronate, GGPP and FPP at the indicated concentrations for 72 h. Thereafter, cells were detached with 0.001% pronase E and the number of viable cells was assessed with Casy cell counter (Schaerfe Systems, Germany). Each experiment was performed in quadruplicate and experiments were carried out twice.

2.4. Measurement of caspase activity

Caspase 3/7 and caspase 8 activities were measured by using the Caspase-Glo 3/7 and Caspase-Glo 8 assay Kit (Promega, Corp., Madison, WI) following manufactures instructions. Briefly, after treatments, cells were lysed and substrate cleavage by caspases was measured by the generated luminescent signal with a 96 multi-well luminometer (Glomax, Promega). Each experiment was performed in quintuplicate and experiments were carried out twice.

2.5. Isolation of nucleic acids and expression analysis by qRT-PCR

DNA and RNA were extracted using a DNA/RNA Isolation Kit (Qiagen, Hilden, Germany) following manufacturers instructions. cDNA was synthesized from 0.5 μg RNA using the 1st Strand cDNA Synthesis Kit (Roche) as described by the supplier. The obtained cDNA was subjected to PCR amplification with a real-time cycler using FastStart SYBR-Green Master Mix (Roche) for the genes *Fas* and *Dnmt1* (primers are shown in Table 1). The qRT-PCR was performed with 45 cycles composed of 30 s denaturation at 95 °C, 30 s annealing at the indicated temperature (Table 1) and 30 s extension at 72 °C after 10 min of initial denaturation at 95 °C. For

Table 1
Primer sequences.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Tm (°C)
Primer for gene expression (mouse and human)			
Dnmt1	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTGG	62
Fas	TATCAAGGAGGCCCATTTTGC	TGTTTCCACTTCTAAACCATGCT	64
Primer for *Fas* promoter methylation assessment			
Mouse	CATACCCACAGGCAGTCTAGA	CAGCCCAGAGTAACTCACTTC	62
Human	CTGACTCCTTCCTCACCCT	CTTCCCCAACTCCGTACT	64
Primer for DNMT1 chromatin immune-precipitation of the *Fas* promoter			
Mouse	CATACCCACAGGCAGTCTAGA	CAGCCCAGAGTAACTCACTTC	62
Human	CTGACTCCTTCCTCACCCT	CTTCCCCAACTCCGTACT	62