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Research paper

The cell cycle arrest and the anti-invasive effects of nitrogen-containing bisphosphonates are not mediated by DBF4 in breast cancer cells

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ABSTRACT

Recent work has shown that a DBF4 analog in yeast may be a target of nitrogen-containing bisphosphonates. DBF4 is an essential protein kinase required for DNA replication from primary eukaryotes to humans and appears to play a critical role in the S-phase checkpoint. It is also required for cell migration and cell surface adhesion. The effects of Pamidronate, risedronate, or zoledronate on cell viability and DBF4 expression were measured via MTT assays and western blotting. In addition, FACS cell cycle analyses and invasion assays were conducted in cells in the presence of nitrogen-containing bisphosphonates to identify any correlations between DBF4 expression and S-phase arrest or anti-invasive effects of the bisphosphonates. Zoledronate transiently down-regulated DBF4 expression in all three cell lines in the first 24 h of the experiment, but after 72 h, DBF4 expression returned to the control levels in all treated cells. Following treatment of the tumor cells with the bisphosphonates, the number of cells in S-phase was increased. Pamidronate and zoledronate showed anti-invasive effects in BT20 cells. The anti-invasive effects of pamidronate, risedronate and zoledronate appeared after 48 h of exposure. In MDA-MB231 cells a reduction of invasiveness was only observed after 72 h of the pamidronate exposure. We finally concluded that the anti-invasive and cell cycle arrest-inducing effects of nitrogen-containing bisphosphonates are not DBF4 mediated, and other mediators are therefore needed to explain the observed complex behaviors.

1. Introduction

Bisphosphonates are a series of synthetic compounds that are widely used in the treatment of bone metabolic diseases associated with high bone resorption, including destructive arthropathy, fibrous dysplasia, heterotopic ossification, osteogenesis imperfecta, and Paget's disease [1]. Bisphosphonates are chemically and enzymatically stable and resemble pyrophosphate analogs characterized by a P–C–P bond [2]. The germinal carbon in this moiety is covalently bonded to two different lateral chains that are usually referred to as the R1 and R2 groups. R1 and R2 confer a pronounced binding affinity for hydroxyapatite and antiresorptive properties in bone, respectively. Based on their molecular structure and mechanism of action, two distinct types of bisphosphonates have been defined. Non-nitrogen-containing bisphosphonates (non-NBPs), such as clodronate and etidronate, are metabolized to form potentially cytotoxic analogs of ATP that accumulate in cells and deactivate osteoclasts, leading to cell death [1]. In contrast, nitrogen-containing bisphosphonates (NBPs), such as alendronate, ibandronate, pamidronate, risedronate, and zoledronate, inhibit intracellular farnesyl diphosphate synthase (FPPS) in the mevalonate pathway, which results in decreased levels of farnesyl diphosphate and geranylgeranyl diphosphate and inhibition of the prenylation of GTP-binding proteins (such as Ras, Rho, and Rac), which is considered to represent the main mechanism of osteoclast activity inhibition and apoptosis promotion [3,4].

Although these bioactive agents are only FDA approved for the treatment of bone metabolic diseases, they have recently gained attention due to their strong apoptosis induction effect in human myeloma cells [5], immunomodulation and stimulation of cytotoxic T-cells targeting tumor cells [4], inhibition of tumor angiogenesis [3], inhibition of the adhesion of invasive cancer cells to bone matrices [6], inhibition of cell invasion and proliferation, and

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promotion of cell cycle arrest. Significant progress has been made in uncovering the mechanisms underlying these anticancerous and immunomodulatory properties. Induction of caspase activity (especially caspase-3 or caspase-7), progressive decreases in the bcl2/bax ratio, and inhibition of the mevalonate pathway are important apoptotic mechanisms associated with the effects of bisphosphonates on tumor cells [4,5]. Interestingly, NBPs down-regulate vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) expression and show potential anti-angiogenetic effects [7]. However, little is known about the molecular mechanisms underlying the anti-invasive and cell cycle arrest-inducing effects of NBPs, and it appears that more complex pathways may be involved. A large-scale experiment examining different yeast mutants in the presence of sub-lethal doses of some NBPs revealed the YDR052C protein (yeasts analog of human DBF4) as a secondary cellular target for these agents [8]. DBF4 is a regulatory subunit of the Cdc7 serine/threonine-specific protein kinase, which is a checkpoint effector enzyme for the initiation of DNA replication and entry into S-phase of the cell cycle [9]. Cdc45 and MCM proteins that bind to replication origins and activate the replication initiation complex are phosphorylated by Cdc7-DBF4. Cdc7 is consistently expressed during the cell cycle, while DBF4 decreases during G1 phase and increases during S and G2 phases. Therefore, up-regulation of DBF4 promotes S-phase [10]. It has also been demonstrated that DBF4 phosphorylates integrin and that depletion of DBF4 kinase reduces cell attachment to collagen-I-coated surfaces in some cells. DBF4 also plays a critical role in cell adhesion and migration, possibly through regulation of actin cytoskeleton arrangement [11]. Although yeast experiments have shown that the anti-invasive and S-phase arrest-inducing effects of NBPs may be mediated via the YDR052C protein, further studies are needed to confirm these mechanisms in human cells. Considering all of these findings, the present study focuses on analysis of the relationship between NBP treatment and DBF4 expression levels in three different human cell lines and the potential consequences of the biological effects of NBPs on the cell cycle and migration behavior.

2. Materials and methods

2.1. Chemicals and media

RPMI-1640 medium, trypsin, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). Pamidronate, risedronate, zoledronate, monoclonal anti-human β-actin (mouse IgG1), monoclonal anti-human DBF4 (goat IgG) antibodies, and an HRP-conjugated secondary antibody (goat anti-mouse IgG) were obtained from Santa Cruz biotechnology (California, USA). Complete Mini Protease Inhibitor Cocktail Tablets™ came from Roche Applied Science (Mannheim, Germany). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Cell lines and cell proliferation assay

The BT20, MDA-MB231, and T47D human epithelial breast cancer cell lines were purchased from the Pasteur Cell Bank, Iran. The cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at a constant temperature of 37 °C in a humidified CO_2 incubator. Proliferation characteristics were determined according to methods used in previous studies by our group. Briefly, 1000 cells were seeded into each well of 96-well plates in 200 μl of growth medium. The culture medium was refreshed every 2 days. Cell growth was assessed during a 7-day incubation period using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 3 h of incubation with MTT (5 mg/ml), the production of formazan crystals was measured using the TECAN microplate reader at 650 nm. All experiments were run three independent times in triplicate, and the results were expressed as the mean values ± SE [12,13].

2.3. Cell cytotoxicity assay

The cells were cultured at a density of 1000 cells per well in 96-well plates and incubated at 37 °C for 24 h. A stock solution of 10 mM pamidronate, risedronate, and zoledronate in 1× phosphate buffered saline (PBS) was prepared and stored in aliquots at −20 °C. The stock was freshly diluted with culture medium before use and added to microplates at defined concentrations from 0 to 100 μM. Cell viability was evaluated after a 5-day incubation of the treated cells using MTT assays. The IC_{50} was determined as the drug concentration that reduced the surviving fraction of cells in each well by 50% compared to the control. All experiments were performed three independent times in triplicate, and the results were expressed as the mean values ± SE [12,14].

2.4. Protein extraction and quantification

The cancer cell lines were cultured in 6-well plates at a density of 6×10^5 cells/well and incubated at 37 °C for 24 h. The cells were then treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h, followed by being washed twice with cold PBS. Next, 300 μl of RIPA lysis buffer (5 mM EDTA, 150 mM NaCl, 1% v/v Triton X-100, 0.1% w/v SDS, 50 mM Tris–HCl pH 8.0 supplemented with a Complete Mini Protease Inhibitor Cocktail Tablet™) was added to approximately 10^6 exponentially growing cells, followed by incubation on ice for 30 min. Cellular lysates were scraped and collected from culture plates, after which they were centrifuged at 14000× *g* for 10 min, and the supernatant (containing total intracellular proteins) was isolated. The supernatant protein content was measured with the BCA Protein Assay Kit® (Thermo Scientific, Rockford, IL, USA), according the manufacturer's protocol, at 562 nm using serial dilutions of human serum albumin as the standard. Unless otherwise specified, all protein extraction procedures were carried out at 4 °C. Finally, the protein samples were stored at −80 °C until further use [15].

2.5. SDS-PAGE and western blotting analysis

The equivalent of 50 μg of each protein sample was mixed with 6× loading buffer (300 mM DTT, 20% v/v glycerol, 4% w/v SDS, 0.3% w/v bromophenol blue, and 120 mM Tris–HCl pH 6.8), heated for 2 min at 95 °C, and then centrifuged at 12000× *g* for 1 min at 4 °C. The samples were next loaded into the wells of a 12% SDS-PAGE gel and electrophoresed in running buffer (190 mM glycine, 0.1% w/v SDS, 25 mM Tris–HCl pH 8.0) for 1.5 h (90 V). The separated proteins were electro-blotted onto a 0.45 μm nitrocellulose membrane (Portran®, Whatman, Germany), stained with Ponceau-S, and washed twice with TBS-T (TBS containing 0.5% v/v tween-20). The nitrocellulose membranes were pre-blocked with TBS-T containing 5% skimmed milk powder for 1 h at room temperature to reduce nonspecific primary antibody binding. Following three washes with TBS–T, the blots were incubated with a diluted mouse monoclonal antibody directed against either human DBF4 (1:600) or human β-actin (1:600) in TBS-T supplemented with 5% skimmed milk overnight at 4 °C. Then, the membranes were treated with the corresponding HRP-conjugated goat anti-mouse IgG (1:5000) in the same supplemented buffer for 1 h at room temperature. The protein-antibody complexes were visualized using a luminol-based