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Zoledronic acid affects the cytotoxic effects of *Chlamydia pneumoniae* and the modulation of cytokine production in human osteosarcoma cells

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ABSTRACT

Chlamydia pneumoniae is an obligate intracellular Gram-negative bacterium that causes persistent infections with a tendency to chronicize, which might motivate the resistance of chlamydiae to some commonly used antibiotics. The bisphosphonates are an emerging class of drugs mostly used in the palliative care of cancer patients to inhibit proliferation and metastasis of cancer cells but their role in modulating immune responses remains unknown. We investigated the in vitro activity of a highly potent bisphosphonate, zoledronic acid, on the cytotoxic effects of *C. pneumoniae* in human SaOS-2 osteoblast-like cells and the consequent immune response carried out by this cell line. We have reported that zoledronic acid showed a significant anti-proliferative effect on SaOS-2 cell line infected by *C. pneumoniae* in a time- and dose-dependent manner. We have also found that zoledronic acid induced growth inhibition of *C. pneumoniae*. Our data showed that *C. pneumoniae*-infection of SaOS-2 cells induced a significant gene expression of proinflammatory cytokines TNF-α, IL-6, IL-8 and IL-12, detected by RT-PCR, and confirmed by protein release assay. Our results demonstrated that zoledronic acid could facilitate *C. pneumoniae*-mediated immune response, thus reprofiling this traditional anti-tumor drug as a novel immune regulator in promoting host defense against *C. pneumoniae* infection.

1. Introduction

Chlamydia pneumoniae is a ubiquitous Gram-negative bacterium, an obligate intracellular parasite with a biphasic life cycle. Chlamydiae are known to be dependent on host cell metabolites, such as amino acids, purines and lipids, due to their intracellular location, similar to most obligate intracellular pathogens [1,2]. This intracellular habitat also requires other mechanisms in surviving, such as controlling host cell apoptosis [3] and evading host immunodefenses [4]. *C. pneumoniae* is a common respiratory pathogen and is associated with acute and at times chronic respiratory infections [5], and also with chronic inflammatory disease [6]. After acute infection, the intracellular life cycle is characterized by the development of metabolically inert (and thus antibiotic resistant) atypical "persistent" inclusions [7], that can induce persistent infection due to the inability of the host to completely eliminate the pathogen [8]. Problems associated with persistent chlamydial growth include the possibilities that it permits immune evasion, provides a reservoir of bacteria that can be reactivated when conditions in the infected microenvironment become permissive [9], and may underlie the recalcitrance of chlamydiae to some commonly used antibiotics [10]. *C. pneumoniae* survives and replicates within mononuclear phagocytes and in non-phagocyte cells, including epithelial cells and fibroblasts [11]; it may also invade osteoblasts and replicate within them [12]. Many pathogens cause bone diseases, though they have very dissimilar characteristics [13]. The ability of *C. pneumoniae* to persist in monocytes and macrophages and in tissues for long periods, to circumvent bactericidal and oxidative stress mechanisms and to activate the production of adhesion molecules and cytokines suggested that it may participate in the development or progression of certain acute and chronic inflammatory diseases of the bone [14].

The bone is a living tissue that is under constant remodeling through an intricate system that facilitates bone resorption by osteoclasts and bone formation by osteoblasts. Recent observations suggest that activated osteoblasts might also serve as a source of soluble inflammatory mediators [13]. The production of high levels of inflammatory mediators may perpetuate inflammation, with consequent bone destruction [12]. Most antibiotics are designed to kill active bacteria only; therefore alternative compounds are receiving considerable attention for use in medical therapy [15]. Bisphosphonates are proven to be effective in the treatment of benign or malignant skeletal diseases characterized

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by enhanced bone resorption [16]. Some primary cancers have a high propensity to metastasize to bone but the reasons for this remain unclear [17]. Bisphosphonates not only inhibit proliferation and induce apoptosis in cultured cancer cells, but also additionally interfere with adhesion of cancer cells and osteoblasts to the bone matrix and inhibit cell migration and invasion [18].

Zoledronic acid (ZA), the third generation of bisphosphonates, is the most potent member in the bisphosphonate family as a standard treatment for preventing skeletal complications associated with bone metastases [19,20]. It is highly probable that ZA, like other aminobisphosphonates, is rapidly internalized in a number of cell types, mainly in osteoclasts and monocytes [21]. It has been shown that aminobisphosphonates indirectly activate a large number of peripheral gamma delta T cells, triggering the release of pro-inflammatory cytokines which are involved in the development of an acute phase response [22]. Recent evidences suggest that zoledronic acid has direct or indirect anti-tumor effects [19,20]. We hypothesized that ZA may be able to modulate innate immunity by osteoblasts when stimulated with pathogens and we focused specifically the relationship between the involvement of the zoledronic acid in inflammatory cytokine production and its effects on *C. pneumoniae* infection. Since osteoblasts have been shown to play a pivotal role in the pathogenesis of osteoarticular diseases caused by various bacteria [12], we used the human SaOS-2 osteosarcoma cell line with osteoblastic properties.

The purpose of the present study was to investigate the influence of zoledronic acid on the cytotoxic effects of *C. pneumoniae* in human SaOS-2 cells and whether this treatment modulates the secretion of interleukin (IL)-6, IL-8, IL-12 and tumor necrosis factor alpha (TNF-α).

2. Materials and methods

2.1. Cell culture and reagents

The human osteosarcoma cell line SaOS-2 (ATCC) was cultured as a monolayer in a 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Milano, Italy) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

The bisphosphonic acid monohydrate ZA, 1-hydroxy-2-[(1H-imidazol-1-yl)ethylidene], a third-generation bisphosphonate, was kindly provided as the hydrated disodium salt by Novartis Pharma AG (Basel, Switzerland). The neutralized sodium salt of ZA was dissolved in sterile ddH_2O and used at final concentrations of 10, 20 and 40 μM. Stock solutions of ZA were aliquoted and kept at −20 °C for long term storage.

3. Propagation of *C. pneumoniae*

C. pneumoniae (AR39) was propagated in HEp-2 cell monolayers as described by Roblin et al. [23]. In brief, *C. pneumoniae* was inoculated onto a pre-formed monolayer of HEp-2 cells in 35-mm diameter wells. The monolayer was then centrifuged at 1000 *g* for 60 min at 25 °C and incubated at 37 °C with 5% CO_2 for 1 h. Supernatants were replaced with growth medium consisting of RPMI-1640 containing 1 μg/ml cycloheximide. Infected cultures were incubated for 72 h at 37 °C in 5% CO_2. *C. pneumoniae* was harvested by disrupting HEp-2 cells with glass beads followed by sonication and centrifugation at 250 ×*g* to remove cellular debris. Supernatants containing *C. pneumoniae* were further centrifuged at 20,000 ×*g* for 20 min to pellet elementary bodies (EBs). The EB pellet was then suspended in sucrose–phosphate–glutamate buffer, aliquoted and stored at −70 °C [24]. Infectivity titers of chlamydial stocks were evaluated by the titration of the inclusion-forming units (IFUs) per milliliter in HEp-2 cells. These titers were used to determine the infectious doses for the cell line studied. Cell cultures and chlamydial stocks were confirmed to be free of *Mycoplasma* infections using 4,6-diamidino-2-phenylindole fluorescent staining (Sigma-Aldrich S.r.l., Milan, Italy). In addition, contamination with *Mycoplasma* was regularly excluded by *Mycoplasma*-PCR using specific primers (MWG Biotech, Martinsried, Germany).

3.1. In-vitro infection

SaOS-2 cells were seeded onto the coverslips in 24-well plates at a density of 5×10^4 cells/well. Before infection the cells were maintained in a basal medium containing 2% FBS without antibiotics. The cells were then infected with *C. pneumoniae* by centrifugation at 1000 ×*g* for 60 min at a multiplicity of infection (MOI) of 4 IFU/cell (a preliminary study showed this MOI to be the optimum rate) and incubated for 24, 48 and 72 h. For some experiments, SaOS-2 cells were pre-treated with ZA for 24 h and then infected with *C. pneumoniae* and determinations were usually performed at 72 h post-infection because this time of infection was found to be the best in preliminary experiments. The count of chlamydial IFU was evaluated as described by Salin et al. [25]. In brief, at indicated times, the medium was removed from the wells and the coverslips were washed twice with PBS and fixed in methanol for 10 min. The coverslips were allowed to dry and the chlamydial inclusions were stained with fluorescein isothiocyanate (FITC)-conjugated anti-MOMP monoclonal antibody (Dako Cytomation, Milan, Italy), according to manufacturer's instructions. The stained inclusions were examined under a fluorescence microscope (AxIOSKop2 Zeiss; Carl Zeiss, Milan, Italy) with a 400× magnification. The amount of the formed inclusions was counted from four eye fields of each coverslip and calculated using the following formula: (inclusions in control − inclusions in treated sample) / inclusions in control × 100.

3.2. Cell proliferation and cell cytotoxicity assays

To study the effects of ZA on cell proliferation and cytotoxicity, SaOS-2 cells were plated at 5×10^4 cells/well in 96-well plates and allowed to grow in the growth medium at 37 °C in CO_2 for 24 h. The cells were then washed once in SRF medium and incubated for another 24 h. They were treated or not (positive control) with the indicated concentrations of ZA for 24 h and then infected by *C. pneumoniae* (MOI = 4) for 72 h. For time-response experiments a ZA concentration of 20 μM was used. Cell proliferation was determined at 24, 48 and 72 h after infection using the MTT assay (3-[4.5-dimethyl-2.5 thiazolyl]-2.5 diphenyl tetrazolium bromide; Sigma-Aldrich S.r.l.) based on the cytoplasmic enzyme activity present in viable cells as described by Hirono et al. [26].

In addition, the cytotoxicity on the untreated, treated and *C. pneumoniae* infected cells was determined by the measurement of the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. Extracellular release of LDH was indicative of cell lysis, as described by Dolfini et al. [27]. Briefly, 50 μl aliquots of cell supernatants were mixed with 25 μl LDH reagent (Sigma-Aldrich) and incubated at room temperature for 30 min. The LDH activity was calculated by measuring the increase in absorbance at 490 nm and was expressed as a percentage of the control value. The experiments were repeated at least three times, and the data were expressed as the mean ± SD.

3.3. C. pneumoniae growth inhibition by ZA

In order to analyze whether ZA treatment could induce growth inhibition in *C. pneumoniae*, SaOS-2 cells were seeded in 96-well plates (Corning Costar, Milan, Italy) at a density of 5×10^5 cells/ml; after 24 h the culture medium was aspired and replaced by fresh FBS-free DMEM containing ZA at concentrations of 10, 20 and 40 μM. The culture medium with or without (positive control) the drug remained in contact with the cells in a humidified incubator containing 5% CO_2 and 95% air at 37 °C for 24 h; cells were then incubated with *C. pneumoniae*