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Vitamin D-dependent cathelicidin inhibits *Mycobacterium marinum* infection in human monocytic cells

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ABSTRACT

Background: 1α,25-Dihydroxyvitamin D3 (1,25(OH)2D3) up-regulates the production of human cathelicidin antimicrobial peptide (CAMP) from monocytes/macrophages infected with *Mycobacterium tuberculosis* (*M. tbc*). CAMP facilitates the co-localization of autophagolysosomes with *M. tbc*, promoting the antimicrobial activity of monocytes. *Mycobacterium marinum* (*M. marinum*) is an acid-fast bacillus that causes less severe granulomatous skin lesions compared with *M. tbc*.
Objective: We investigated whether autophagic antimicrobial activity is promoted by 1,25(OH)2D3 or C-terminal of cathelicidin LL-37 in human monocytes upon infection with *M. marinum*.
Methods: Human monocytes (THP-1) were infected with *M. marinum*. Effects of simultaneous treatments of 1,25(OH)2D3, exogenous LL-37 peptide, autophagolysosome inhibitors, 3-methyladenine or chloroquine, were examined.
Results: CAMP was strongly induced by adding 1,25(OH)2D3 to the culture of THP-1 cells. In the absence of 1,25(OH)2D3 *M. marinum* infection alone did not induce CAMP, however, simultaneous addition of 1,25(OH)2D3 to *M. marinum* infection accelerated CAMP production more than 1,25(OH)2D3 alone. Proliferation of *M. marinum* was markedly decreased in the presence of 1,25(OH)2D3 or exogenous LL-37 in THP-1 cells. Co-localization of CAMP with autophagolysosome was evident in 1,25(OH)2D3 and LL-37 treated THP-1 cells after *M. marinum* infection. Autophagolysosome inhibitors abrogated the antimicrobial effects of 1,25(OH)2D3 and exogenous LL-37 against *M. marinum* infection in THP-1 cells.
Conclusions: Human monocytic cells, whose CAMP production is up-regulated by 1,25(OH)2D3-vitamin D receptor pathway, accelerate antimicrobial function of autophagolysosome in *M. marinum* infection.

1. Introduction

The human cathelicidin antimicrobial peptide (CAMP) has been shown to exhibit broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacterial species. CAMP gene is a direct target of the vitamin D receptor (VDR) and is strongly up-regulated in various cells such as neutrophils, macrophages and epithelial cells by 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3), an active form of vitamin D [1,2]. CAMP becomes its mature extracellular form of 37 amino acid peptide, LL-37, after truncation by protease, and executes its antimicrobial activity directly against invading pathogens.

It has been shown that CAMP plays intracellular roles in addition to direct antimicrobial activity against pathogens. Jo et al. have recently shown that 1,25(OH)2D3 can up-regulate the production of CAMP from monocytes/macrophages infected with *Mycobacterium tuberculosis* (*M. tbc*), and CAMP induces autophagy and facilitates the co-localization of autolysosomes with *M. tbc* to promote the antimicrobial activity of monocytes [3]. Autophagy is a generic term for all pathways by which cytoplasmic materials are delivered to the lysosome in animal cells or the vacuole in plant and yeast cells [4,5]. Autophagy occurs when an autophagosome (a double-membrane vacuole) containing cytoplasmic material, fuses

Abbreviations: CAMP, cathelicidin antimicrobial peptide; VDR, vitamin D receptor; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; *ATG*, autophagy-related genes; CQ, chloroquine; 3-MA, 3-methyladenine.

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with a lysosome to deliver sequestered material for lysosomal degradation [6], and multiple autophagy-related genes (*ATG*) proteins govern autophagosome formation [7]. Growing evidence suggests that autophagy contributes to the intracellular killing of *M. tbc* by facilitating phagolysosome fusion and thereby providing a mechanism to counteract the ability of *M. tbc* to evade the host response [8–10].

Mycobacterium marinum (*M. marinum*) is a nontuberculous photochromogenic mycobacterium species belonging to group I of Runyon's classification [11], and causes granulomatous skin lesions. The cutaneous infection caused by *M. marinum*, which mostly affects those who own aquariums or are in contact with fish, is the most common. The lesion, usually at the hand or forearm, is initially nodular but may subsequently ulcerate, whereas the sporotrichoid form is characterized by small nodules along lymphatic ducts [12]. *M. marinum* has been increasingly studied as a model of *M. tbc* due to its relative safety and its shared mechanisms of pathogenesis [13–15]. However it still remains to be determined whether 1,25(OH)2D3 and/or LL-37 can augment antimicrobial activity against *M. marinum* infection like against *M. tbc* infection as recently shown. Here we show that antimicrobial activity is promoted by autophagy via 1,25(OH)2D3 or LL-37 in human monocytes infected with *M. marinum*.

2. Materials and methods

2.1. Cells and reagents

THP-1 cells and U937 cells were purchased (American Type Culture Collection) and maintained in RPMI 1640 (Wako, Osaka, Japan) with 10% FBS (GIBCO, Carlsbad, CA, USA). Autophagy-related gene 5 deficient (*ATG5*$^{-/-}$) MEF cells were obtained from Riken BioResource Center, Cell Bank (Ibaraki, Japan) and maintained in DMEM with 10% FBS [16]. 1,25(OH)2D3 (BIOMOL International) was added to the culture at 10 nM and synthetic LL-37 peptide (Innovagen, Lund, Sweden) was added at 10 μg/ml. Autophagy antagonists 3-methyladenine (3-MA) and chloroquine (CQ) (Sigma–Aldrich, St Louis, MO, USA) were added to the culture at 1 μg/ml and 5 μM, respectively.

2.2. Pathogens and colony-forming unit (CFU) assay

M. marinum strain NJB0419 was obtained from Japan Anti-Tuberculosis Association (Tokyo, Japan) and cells were infected with *M. marinum* at multiplicity of infection (MOI) of 1–10. The efficiency of infection was determined by Ziehl-Neelsen staining. Infected cells were treated with 1,25(OH)2D3 or LL37 in triplicate wells, and then collected between 1 and 3 days. Cells were centrifuged at 2000 rpm for 5 min to form a pellet and then the supernatant was aspirated. Intracellular *M. marinum* were obtained by lysing the cells with 0.5% Triton X-100 in PBS. *M. marinum* isolates were 10-fold serially diluted and plated on Middlebrook 7H10 (BD Bioscience, San Jose, CA, USA) agar plates supplemented with 10% OADC Enrichment, then incubated at 30 °C for 10 days. Colonies formed were counted as CFU for quantification of the mycobacteria. *M. marinum* optical density (OD_{570}) is measured by Model 680 Microplate Reader (Bio-Rad, CA, USA).

2.3. Isolation of total RNA and quantitative RT-PCR

Total RNA was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 μg of the total RNA using a SuperScript™ III First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR Premix DimerEraser™ (TaKaRa, Siga, Japan) in a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The gene-specific primer sequences are shown in Table 1. β-Actin was amplified as an internal control each time qRT-PCR was performed, and the ΔΔCt method was employed to quantify the relative amounts of transcripts. The Student *t*-test was used for statistical analyses. A p value < 0.05 was considered statistically significant.

Table 1
Primer sequences of assayed genes.

CAMP	Forward	5′-GGA CCC AGA CAC GCC AAA-3′
	Reverse	5′-GCA CAC TGT CTC CTT CAC TGT GA-3′
IL-12p40	Forward	5′-CAG CTC GCA GCA AAG CAA-3′
	Reverse	5′-GAC GCC ATT CCA CAT GTC ACT-3′
TNF-α	Forward	5′-TCT CGA ACC CCC GAG TGA CA-3′
	Reverse	5′-GGC CCG GCG GTT CA-3′
IFN-β	Forward	5′-TGC TCT CCT GTT GTG CTT CTC C-3′
	Reverse	5′-CAT CTC ATA GAT GGT CAA TGC GG-3′
β-Actin	Forward	5′-AAG GGA CTT CCT GTA ACA ATG CA-3′
	Reverse	5′-CTG GAA CGG TGA AGG TGA CA-3′

2.4. Enzyme-linked immunosorbent assay (ELISA)

Interleukin (IL)-12/23 p40, interferon (IFN)-β or tumor necrosis factor (TNF)-α in the supernatants of treated cells was measured using commercially prepared ELISA plates according to the manufacturer's suggestion (IL-12/23 p40 and TNF-α, R&D Systems, Minneapolis, MN, USA; IFN-β, Peprotech, Rocky Hill, NJ, USA).

2.5. Western blot

2×10^5 cells were lysed and separated on 15% SDS–PAGE gels and transferred onto a polyvinylidene difluoride membrane as described previously [17]. Membranes were blocked in PBS containing 5% skim milk at room temperature for 1 h. The specific proteins were determined by incubation with specific antibodies against human β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VDR or LL-37 (1:200, Santa Cruz Biotechnology) at 25 °C for 1 h. After washing, the membrane was incubated in a 1:3000 dilution of a secondary antibody (sheep anti-mouse IgG-HRP conjugate; GE Healthcare, Little Chalfont, UK) at room temperature in the washing buffer (PBS containing 0.5% Tween 20) for 30 min. The protein bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare). Densitometry LAS-3000 software (FUJIFILM, Tokyo, Japan) was used to quantify the LL-37 protein levels, and the levels were normalized to β-actin.

2.6. Immunofluorescent staining

Cells were seeded onto glass cover slips in 24-well plates. Cells were fixed in 3.7% paraformaldehyde, incubated in 50 mM glycine for 5 min, permeabilized and blocked with 1.5% BSA for 30 min. Immunostaining was performed using polyclonal anti-LL-37 antibodies (1:1000, Innovagen) or monoclonal anti-LC3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and LAMP-1 antibodies (1:200, Santa Cruz Biotechnology). Secondary Alexa Fluor 488 and 546-conjugated antibodies were obtained from Invitrogen and used by 1:2000. Cells were washed with PBS, and cover slips were mounted using ProLong® Gold antifade Reagent with DAPI (Invitrogen). Fluorescence images were acquired by BIOREVO BZ-9000 (Keyence, Japan). Presented are representative results observed in the majority of cells from several repeats.