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Original article

Proteomic analysis and immune properties of exosomes released by macrophages infected with *Mycobacterium avium*

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Abstract

The role of exosomes shed from *Mycobacterium avium* sp. *paratuberculosis*-infected macrophages in intercellular communication processes was examined. We compared the responses of resting macrophages infected with *M. avium* sp. *paratuberculosis* with those of resting macrophages treated with exosomes previously released from macrophages infected with *M. avium* sp. *paratuberculosis*. Some proteins components of exosomes released from resting macrophages infected with *M. avium* sp. *paratuberculosis* showed a significantly differential expression compared with exosomes from uninfected-macrophages. Both *M. avium* sp. *paratuberculosis* and exosomes from infected-cells enhanced the expression of CD80 and CD86 and the secretion of TNF-α and IFN-γ by macrophages. This suggests that exosomes from infected macrophages may be carriers of molecules, e.g. bacterial antigens and/or components from infected macrophages, that can elicit responses in resting cells. Two-dimensional analysis of the proteins present in exosomes from *M. avium* sp. *paratuberculosis*-infected macrophages compared with those from resting cells resulted in the identification by MALDI-TOF/TOF mass spectrometry of the following differentially expressed proteins: two actin isoforms, guanine nucleotide-binding protein β-1, cofilin-1 and peptidyl-prolyl cis–trans isomerase A. The possible relevance of the changes observed and the biological functions of the proteins differentially present are discussed.

Keywords: *Mycobacterium avium*; Macrophage; Exosomes; 2-DE; MALDI-TOF/TOF MS

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) has become a serious public health problem all around the world, China being among the countries worst affected. The body's fight against *M. tuberculosis* infection requires the development of a Th1-type CD4+ T-cell response and activation of alveolar macrophages, leading to the formation of lung granulomas [1]. *Mycobacterium avium* (*M. avium*) shares with *M. tuberculosis* a slow growth rate and an ability to generate granulomas, and is itself a disease-causing agent [2–4]. *M. avium* and *M. tuberculosis*-containing phagosomes share important features such as a restricted fusogenicity with endosomal/lysosomal compartments [5–7] and an impaired acidification [8,9]. Because of its eliciting a response similar to that against *M. tuberculosis* and taking also safety into account, *M. avium* is a more convenient than *M. tuberculosis* for studies like the present one involving procedures such as cell fractionation, ELISA and FACS analysis.

Macrophages are the main cells that, when activated, can kill mycobacteria. They constitute the first barrier preventing dissemination of these bacteria *in vivo*. At the same time, macrophages can also act as a *M. tuberculosis* reservoir in the body [10]. Cellular immunity to mycobacteria requires a coordinated response between the innate and adaptive arms of the immune system, resulting in a type 1 cytokine response. After mycobacteria are phagocytosed, their intracellular killing can be modulated by cytokines such as IFN-γ, TNF-α, IL-10, IL-1 and IL-12 [11,12]. For instance, these cytokines

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can regulate NO and ROS generating systems crucial for bacterial killing. *M. tuberculosis* engulfment by macrophages is followed by their intraphagosomal processing, which results in the generation of short bacterial peptides. These peptides, when transported to the cell surface bound to MHC-I and MHC-II molecules, can activate CD8+ and CD4+ T lymphocytes able to recognize specifically *M. tuberculosis* antigens. Lymphocytes can also be induced to secrete IFN-γ, IL-12, TNF-α and other cytokines with immunomodulatory functions [13]. Besides, CD8+ T cells can themselves kill *M. tuberculosis* [14]. CD80 (B7-1) and CD86 (B7-2) are B-cell and macrophage co-stimulatory T lymphocyte-activating molecules involved in the regulation of immune function by interacting with and priming T cells [15]. These molecules participate in co-stimulatory signals necessary for T cell activation and survival during immune responses, acting as ligands for the molecules CD28 and CTLA-4 on the T cell surface [16].

Exosomes are small membranous vesicles generated by inward budding of late endosomes, which results in the formation of multivesicular bodies (MVBs) in the cell cytosol. They are subsequently exocytosed to the extracellular space by fusion with the plasma membrane [17]. They participate in immuno-stimulation and protein secretion events [18,19]. In general, exosomes act as molecule carriers during immune cell–cell communication [20]. Exosomes secreted by antigen-presenting cells are rich in MHC-I/II and co-stimulatory molecules and can play immune stimulatory roles such as promoting the proliferation of T lymphocytes [21]. Knowledge on the protein composition of exosomes can suggest functions of these extracellular vesicles. For instance, Hsp70-containing exosomes show a pro-inflammatory action [22] and exosomes containing *M. avium* glycopeptidolipids (GPLs) transfer them from infected to non-infected macrophages, which results in a toll-like receptors-dependent pro-inflammatory response [23]. Exosomes secreted by macrophages infected with *M. tuberculosis* contain proteins such as antigen 85A, B and C, which are presently being tested as boosters in clinical vaccines [15]. Therefore, the elucidation of exosome components in relationship with mycobacterial immunity could add relevant information on how these cell-derived vesicles act as transferring vectors between cells.

2. Materials and methods

2.1. Reagents

Phorbol 12-myristate 13-acetate (TPA) was purchased from Sigma and dissolved in DMSO, kept at −20 °C and used at a final concentration of 0.1 mmol/l. SYBR Green I fluorescent dye was bought from Takara (Da Lian, China). ELISA detection kits for TNF-α, IFN-γ were purchased from KuangBo biotechnology (Bei Jing, China). CD80-FITC and CD86-PE antibodies were purchased from Biolegend (California, USA). Immobilized pH gradient strips and DeStreak rehydration solution were bought from GE Healthcare (USA).

2.2. Macrophage cultures

The human acute monocytic leukemia cell line THP-1 was purchased from the Center for Type Culture Collection of Wuhan University. Cells were cultured in wells or flasks at 37 °C under 5% CO_2, in RPMI 1640-GlutaMAX™ containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. Differentiation of these cells into macrophage-like cells was induced with 0.1 mmol/l TPA for 24 h.

2.3. M. avium *growth*

M. avium sp. *paratuberculosis*(*M. avium* spp called *M. avium* in this paper) was obtained from the Chinese Center for Disease Control and Prevention. Bacteria were grown on Middlebrooks 7H9 plates as previously described [24] for 4 weeks, at 37 °C. They were harvested by scraping, with 0.9% NaCl as vehicle, and concentrations were calculated according to McFarland Standards method, then were adjusted to 7.5×10^9/ml.

2.4. M. avium *infection of macrophages*

Macrophages were cultured at 1×10^6 cells (in 1 ml culture medium) per well in six well plates infected with *M. avium* at a ratio of 100 bacteria/cell and then cultured for the times indicated. Supernatants were then collected and used for cytokine analysis and exosome isolation. The uninfected or infected cells were washed with PBS and used to analyze genes and proteins of CD80, CD86.

2.5. Acid-fast staining of infected macrophages

Macrophages infected with *M. avium* (100 bacteria/cell) for 0, 12, 24 and 48 h were heated for 5 min with carbol fuchsin, washed with water, destained with 3% hydrochloric acid in ethanol for 1 min, rinsed with water, stained with alkaline methylene blue solution for 1 min and washed with water. Cells were then microscopically observed.

2.6. Exosome isolation

Exosomes were isolated according to published reports [23], with some modifications. All procedures were carried out at 4 °C. Cell culture supernatants from either uninfected or *M. avium*-infected (48 h) cells were centrifuged at 18,000 *g* for 20 min in order to remove cells and cell debris. The supernatants were first centrifuged at 46,300 *g* for 45 min to remove bacteria and finally at 84,000 *g* for 60 min. The pellets therefrom were resuspended with 200 μl of 9% sucrose containing protease inhibitors and centrifuged at 100,000 *g* for 45 min. The pellets (exosomes) were resuspended with 50 μl of 9% sucrose containing protease inhibitors and stored at −80 °C. The term (+)exosomes describes exosomes obtained from *M. avium*-infected macrophages and the term (−)exosomes describes exosomes from non-infected macrophages.