Molecular Immunology 55 (2013) 220–230

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Lymphocyte-derived microparticles induce bronchial epithelial cells' pro-inflammatory cytokine production and apoptosis

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ARTICLE INFO

Article history:
Received 23 November 2012
Received in revised form 24 January 2013
Accepted 30 January 2013
Available online 19 March 2013

Keywords:
Airway epithelium
Tumor necrosis factor-α
Interleukin-6
Interleukin-8
Caspase-3, -8, -9

ABSTRACT

Objective: The aim of this study was to determine if human CEM (human lymphoblastoma) T cell-derived microparticles (LMPs) could directly induce human bronchial epithelial cells (BECs) apoptosis and cytokine production. We also tested if LMPs phagocytosis by BECs played a role in mediating these effects.
Methods: We generated LMPs from CEM (human lymphoblastoma) T cells to investigate their effects on a human BEC cell line (16HBE) in vitro.
Results: BECs (16HBE cells) incubation with LMPs resulted in significant production of inflammation-associated cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-8, in a dose- and time-dependent manner. LMPs also induced increased activities of caspase-3, caspase-8, and caspase-9 in BECS, which resulted in increased BECs apoptosis as assessed by flow cytometry (Annexin V and propidium iodide staining) and transmission electronic microscopy (TEM). Interestingly, LMPs effects on BECS were inhibited by the phagocytosis inhibitors cytochalasin D and chloroquine.
Conclusions: These results suggest that phagocytosis plays an important role in mediating the effects of LMPs on BECs. Thus, increased LMP concentrations may contribute to increased respiratory inflammatory responses and innate immune response maintenance in airway epithelium after LMPs engulfment by endothelial cells.

1. Introduction

Microparticles (MPs) are submicron-sized membranous vesicles (diameters between 0.1 and 1 μm) that contain cell surface proteins and cytoplasmic components and are shed by cells under stress, such as activated and apoptotic cells (Burnier et al., 2009; Hugel et al., 2005). The levels of circulating microparticles, such as leukocyte-derived MPs (LMPs), are significantly increased in patients with pulmonary hypertension (Amabile et al., 2008). Increased levels of pro-coagulant alveolar microparticles are found in alveolar edema samples or bronchoalveolar lavage fluids (BALF) from patients with acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and COPD (Bastarache et al., 2009; Mutschler et al., 2002). Thus, MPs appear to be involved in the pathogenesis of numerous respiratory diseases, particularly those characterized by chronic inflammation.

Damage to bronchial epithelial cells (BECs) that causes ciliary dysfunction is a key property of the pathogenesis of airway inflammation and is thought to be an important contributor to the development of chronic inflammatory pathological changes and airway hyper-responsiveness. A variety of cells types, such as lymphocytes, other leukocytes, platelets, and endothelial cells, can access the bronchi and alveolar spaces. Thus, these cells could be the sources of intra-bronchial and intra-alveolar MPs that accumulate on the surfaces of BECs and function as activating molecules to induce functional changes (Buesing et al., 2011).

There is considerable evidence that substantially increased numbers of circulating LMPs are associated with several pathological conditions of immune and inflammatory disorders, such as rheumatoid arthritis, malaria, septicemia, HIV infection, and preeclampsia (Aupeix et al., 1997; Berckmans et al., 2005; Couper et al., 2010; Meziani et al., 2006; Mostefai et al., 2008). A proteomics

Abbreviations: MPs, microparticles; LMPs, leukocyte-derived MPs; BALF, bronchoalveolar lavage fluids; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BECs, bronchial epithelial cells; 16HBE, human bronchial epithelial cells; CEM-T, human lymphoblastoma cells; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiI-LMPs, DiI-labeled LMPs; TEM, transmission electron microscopy; pNA, p-nitroanilide; PI, propidium iodide; sIL-1Ra, the secreted form of the IL-1 receptor antagonist; MCP-1, monocyte chemoattractant protein 1; PS, phosphatidylserine; PSR, phosphatidylserine receptor; LDLR, low-density lipoptrotein receptors.

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http://dx.doi.org/10.1016/j.molimm.2013.01.017

analysis of LMPs from malignant lymphocytes identified 413 proteins, including 117 membrane proteins, many of which were associated with various pathologies (Miguet et al., 2006). Thus, LMPs are biomarkers and vectors that carry biological information between cells (Morel et al., 2011).

LMPs derived from human Jurkat T-lymphoma cells are rapidly engulfed by RAW 264.7 mouse macrophages and consequently induce macrophage apoptosis (Distler et al., 2005a). We also observed that LMPs derived from human CEM (human lymphoblastoma) T cells strongly reduced human umbilical vein endothelial proliferation without inducing endothelial cell death (Yang et al., 2008). LMPs also caused a dose-dependent inhibition of Lewis lung carcinoma cells growth, although these effects were associated with increased cellular apoptosis (Yang et al., 2010). Interestingly, resting and cytokine-stimulated human BECs can selectively recognize and engulf apoptotic eosinophils (Sexton et al., 2004; Walsh et al., 1999). However, no studies have investigated the effects of LMPs on BECs with regard to these cells' phagocytic activities.

Previous studies have also shown that BECS played key roles in immune regulation during the development of bronchial inflammation that was linked to either contact with inflammatory cells or activation by inflammatory mediators in response to pathogen-associated molecular pattern molecules (Bals and Hiemstra, 2004). Monocyte/macrophage-derived microparticles induced increased expression of IL-8 in human bronchial epithelial and alveolar cells (Cerri et al., 2006; Neri et al., 2011). Distler et al. (2005b) confirmed that LMPs induced the expressions of various inflammatory mediators, including IL-6, IL-8, MCP-1, MCP-2, and MMPs, in synovial fibroblasts in rheumatoid arthritis.

Thus, in this study we investigated if human CEM (human lymphoblastoma) T cell-derived microparticles (LMPs) could directly induce apoptosis and cytokine production by human BECs. We also examined if LMPs phagocytosis by BECs played a role in mediating these LMP effects. Our results show that LMPs phagocytosis by BECs is directly related to BECs apoptosis and their production of pro-inflammatory cytokines.

2. Materials and methods

2.1. Cell culture

Human bronchial epithelial cells (16HBE) and human lymphoblastoma cells (CEM-T) were purchased from the ATCC (Manassas, VA, USA) and cultured in RPMI-1640 Medium (Gibco BRL, Long Island, NY) supplemented with 10% heat inactivated FBS (Hyclone laboratories, South Logan, UT, USA), 2 mM L-glutamine, 1% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells between passages 4–9 were used.

2.2. LMPs generation

LMPs were released into the culture supernatants of CEM-T cells after 24 h stimulation with actinomycin D (5 μg/ml; Sigma Chemical Co., St. Louis, MO, USA). LMPs were isolated and characterized as described previously (Yang et al., 2008, 2010). Briefly, a supernatant was obtained after removing cells by centrifugation at 750 × *g* for 5 min. The supernatant was centrifuged at 1500 × *g* for 15 min to remove large cell fragments. After ultracentrifugation at 12,000 × *g* for 50 min, LMPs in the supernatant were washed twice in PBS, resuspended in Dulbecco's phosphate buffered saline, and assayed for protein concentration using a Bio-Rad protein assay. Cy3-Annexin V (Sigma Chemical Co., St. Louis, MO, USA) binding and the sizes of LMPs were determined by flow cytometry (FACSCalibur, BD Bio-sciences), as described previously (Shet et al., 2003). An event discrimination threshold was set for the side-scatter channel at the lowest channel allowed and size gates were set using 1 μm latex beads (Sigma). When kept at 4 °C under sterile conditions in PBS, isolated LMPs were stable for at least 3 weeks with regard to their ability to induce cytokine production by human epithelial cells. The fluorescent membrane cell linker 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Sigma) was added to CEM-T cells 24 h before actinomycin D treatment to generate labeled LMPs (DiI-LMPs) according to the supplier's protocol.

2.3. Microparticle staining and fluorescence microscopy

DiI-labeled LMPs (DiI-LMPs) were freshly isolated. For conventional fluorescence microscopy, epithelial cells were seeded on cover slips in 6-well plates and incubated with 20 μg/ml of DiI-LMPs for different times to determine specific uptake versus non-specific binding. Cells were then washed three times, after which images were captured with an Olympus I × 51 inverted microscope equipped with an Olympus DP70 digital camera (Olympus Optical, Tokyo).

For confocal fluorescence microscopy, 20 μg/ml of DiI-LMPs were added to epithelial cells that had grown on round cover slips in 24 well plates. To inhibit phagocytosis, 20 μM of cytochalasin D (Fluka, Buchs, Switzerland) or 20 μg/ml of chloroquine (Sigma) was added 2 h before LMPs treatment. After incubation for the indicated times, epithelial cells were fixed with paraformaldehyde and nuclei were stained with DAPI (Sigma). Fluorescence microscopy used a Zeiss LSM510 laser scanning confocal microscope attached to a Zeiss Axiovert 200 microscope using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion lens.

For lysosome marker staining, 5000 HBE cells were seeded into a 35 mm glass culture dish (NEST Biotechnology Co., LTD., Wuxi, China). After cells were attached, DiL-LMPs (20 μg/ml) were added and cultured for 12 h. Then, the medium was removed from the dish and replaced with pre-warmed (37 °C) medium with 0.5 μM LysoSensor LysoTracker® Green DND-26 (L7526, Invitrogen Molecular Probes, Inc., Eugene, OR). Cells were incubated for 30 min at 37 °C, after which the loading solution was replaced with fresh medium. Cells were observed using a laser confocal microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

2.4. Cell growth assay

The effects of different treatments on cell growth were determined by mitochondria-dependent reduction of MTT (Sigma). Cells at ≈60% confluence were incubated for 24 h with either vehicle or the indicated concentrations of LMPs. MTT (0.5 mg/ml in PBS, pH 7.4) was added to the culture medium, followed by incubation for 3 h, after which the medium was aspirated. The formazan product was solubilized with dimethyl sulfoxide (Sigma) and optical density was read on a Bio-RAD microplate reader (Bio-RAD Laboratories, Inc., USA) at 545 nm with a reference wavelength at 690 nm.

2.5. Ki67 immunostaining

16HBE cells were incubated with or without 20 μg/ml of LMPs for 24 h, 48 h, or 72 h. Cells were then fixed with 4% polyoxymethylene, blocked with BSA, treated with 0.2% trixon-100, and stained with a purified anti-Ki67 mouse monoclonal antibody (TA500265; ZSGB-BIO, Beijing, China) at 4 °C overnight. A secondary Ab was Cy5-goat anti-mouse. DAPI was used to stain nuclei. Cells were observed with a laser confocal microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).