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Exosomes derived from calcium oxalate-exposed macrophages enhance IL-8 production from renal cells, neutrophil migration and crystal invasion through extracellular matrix

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ABSTRACT

Deposition of calcium oxalate (CaOx) crystals in renal interstitium is one of the key factors that cause progressive inflammation in kidney stone disease. Macrophages are responsible for elimination of these crystals but their roles to worsen inflammatory process remain under-investigated. This study thus aimed to define roles of exosomes released from macrophages exposed to CaOx crystals in mediating subsequent inflammatory cascades. Macrophages were incubated with or without CaOx monohydrate (COM) crystals for 16 h and their exosomes were isolated. Quantitative proteomics using nanoLC-ESI-Qq-TOF MS/MS revealed 26 proteins with significantly altered levels in exosomes derived from COM-treated macrophages (COM-treated exosomes) comparing to those derived from the controlled macrophages (controlled exosomes). Protein network analysis showed that these altered proteins were involved in cytoskeleton and actin binding, calcium binding, stress response, transcription regulation, immune response and extracellular matrix disassembly. Functional investigations revealed that COM-treated exosomes enhanced IL-8 production from renal tubular cells, activated neutrophil migration, had increased (exosomal) membrane fragility, had greater binding capacity to COM crystals, and subsequently enhanced crystal invasion through extracellular matrix migration chamber. These data indicate that macrophage exosomes play important roles in inflammatory response to COM crystals and may be involved in crystal invasion in the renal interstitium.

1. Introduction

Deposition of calcium oxalate (CaOx) crystals in renal interstitium is one of the key factors that cause progressive inflammation in kidney stone disease [1]. The interstitial inflammation then leads to renal cell necrosis, cytokine/chemokine overproduction, deposition of extracellular matrix (ECM), and ultimately renal fibrosis [2, 3]. Overproduction of cytokines/chemokines, i.e., osteopontin, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), regulated on activation, normal T-cell expressed and secreted (RANTES), and interleukin (IL-8), can further enhance the detrimental effects by recruiting various immune cells into the inflammatory site [4]. Because CaOx crystals are considered as the foreign body, macrophages are apparently the important effector cells in response to CaOx crystals accumulated in the renal interstitium [5, 6]. In addition to the role to eliminate these deposited crystals, vice versa roles of macrophages to further worsen the inflammatory process after exposure to CaOx crystals remain poorly understood and under-investigated. This study thus aimed to define mechanism underlying such inflammatory process triggered by macrophages, particularly roles of exosomes released from macrophages exposed to CaOx monohydrate (COM) (which is the most potent form of CaOx crystals involving in kidney stone formation [7–9]) in mediating subsequent inflammatory cascades.

2. Materials and methods

2.1. Cell culture and macrophage differentiation

Macrophages were derived from U937 human monocytic cell line using phorbol 12-myristate 13-acetate (PMA) (Sigma; St. Loius, MO) for differentiation as previously described [10]. Briefly U937 human monocytic cell line was maintained in complete RPMI 1640 medium

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(Gibco; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Sigma), 60 U/ml penicillin G (Sigma), and 60 mg/ml streptomycin (Sigma) at 37 °C in a humidified incubator with 5% CO_2. U937 cells at a density of 1×10^6 cells/ml were then treated with 100 ng/ml PMA for 48 h (induction phase) and then vigorously washed three times with ice-cold PBS to remove PMA and non-adherent cells, whereas the adherent cells were further maintained as aforementioned for 48 h (recovery phase). The characteristics of macrophages were observed under an inverted phase-contrast microscope (ECLIPSE Ti-S, Nikon; Tokyo, Japan) as previously described [10].

Madin-Darby canine kidney (MDCK) cell line (which displayed many characteristics of MDCK Type II) was maintained in Eagle's MEM medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 60 U/ml penicillin G, and 60 mg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2 as described previously [11, 12].

2.2. CaOx monohydrate (COM) crystal preparation and treatment

COM crystals were prepared as described previously [13, 14]. Briefly, 10 mM $CaCl_2.2H_2O$ was mixed with 1.0 mM $Na_2C_2O_4$ (1:1 v/v) to make their final concentrations to 5 mM and 0.5 mM, respectively, in a buffer containing 10 mM Tris-HCl and 90 mM NaCl (pH 7.4). After incubation at 25 °C overnight, COM crystals were harvested by a centrifugation at 2000*g* for 5 min. The supernatant was discarded, whereas COM crystals were washed 3 times with methanol. After another centrifugation at 2000*g* for 5 min, methanol was discarded and the crystals were air-dried overnight at 25 °C. The typical morphology of COM crystals was examined under an inverted phase-contrast light microscope (Nikon ECLIPSE Ti-S).

The COM crystals were decontaminated by exposure to UV light for 30 min prior to incubation with the cells. After recovery phase, U937-derived macrophages (10×10^6 cells/flask) were vigorously washed five times with ice-cold PBS to remove serum-containing medium and further cultivated in serum-free RPMI 1640 medium with or without 100 μg/ml COM crystals for 16 h, which was the optimal time-point defined for studying macrophage secretome as previously reported [10]. After 16-h incubation, the culture supernatants were harvested and further subjected to exosome isolation as detailed below.

2.3. Exosome isolation by microfiltration and differential centrifugation

The controlled and COM-treated macrophage supernatants were filtrated through 0.22-μm cellulose acetate membrane (Sartorius Stedim Biotech GmbH; Goettingen, Germany) to remove cell debris and apoptotic bodies. Microvesicles and/or larger vesicles were further removed by centrifugation at 10,000*g* and 25 °C for 30 min. Exosomes were then isolated from the remaining supernatants by ultracentrifugation at 100,000*g* and 25 °C for 90 min using an ultracentrifuge (Sorvall; Langenselbold, Germany). The isolated exosomal pellets were washed twice with PBS and resuspended in PBS. The intact exosomes were subjected to functional assays as detailed below. For protein quantification, the isolated exosomes were resuspended in SDT lysis buffer containing 4% SDS, 100 mM dithiothreitol (DTT), and 100 mM Tris-HCl (pH 7.6) at 4 °C for 30 min. Protein concentrations were measured by the Bradford's method using Bio-Rad protein assay (Bio-Rad; Milano, Italy).

2.4. Transmission electron microscopy (TEM)

Exosomes were resuspended in 2% (w/v) paraformaldehyde and loaded onto carbon-Formvar-coated copper grids. The samples were left on the grids for 20 min to adsorb and form monolayers, and were then washed three times with PBS. Thereafter, the grids were fixed with 50 μl of 2% (v/v) glutaraldehyde for 5 min and subsequently washed eight times with distilled water. The grids were contrasted with 50 μl of 4% (v/v) uranyl acetate (pH 7.0) for 5 min and the excess fluid was then removed by filter paper. Finally, the grids were loaded onto a transmission electron microscope (FEI Tecnai T20 TEM Series; Hillsboro, OR) with an operating voltage of 200 kV at a magnification of 89,000×.

2.5. In-solution tryptic digestion by filter-aided sample preparation (FASP) method

Protein samples prepared in SDT lysis buffer were reduced by heating at 95 °C for 5 min. After cooling down at 25 °C, an equal amount of protein (30 μg/sample) was transferred to an Omega Nanosep 10 K device (Pall Corporation; Port Washington, NY), added with 200 μl of 8 M urea in 100 mM Tris-HCl (pH 8.5), and then centrifuged at 14,000*g* and 25 °C for 15 min. This buffer exchange step was repeated one more cycle. The recovered proteins were then alkylated with 100 μl of 50 mM iodoacetamide in 8 M urea/100 mM Tris-HCl (pH 8.5) at 25 °C in the dark using a ThermoMixer® C (Eppendorf; Hauppauge, NY) for 20 min. Thereafter, buffer exchange was performed twice by centrifugation at 14,000*g* and 25 °C for 15 min each using 200 μl of 8 M urea/100 mM Tris-HCl (pH 8.5). The proteins were then finally exchanged into 50 mM NH_4HCO_3 and digested with sequencing grade modified trypsin (Promega; Madison, WI) in 50 mM NH_4HCO_3 at a ratio of 1:50 (w/w) trypsin/protein at 37 °C for 16–18 h in a ThermoMixer® C. The digested peptides were collected by transferring the filter unit to a new collection tube and centrifuged at 14,000*g* and 25 °C for 10–20 min. Trypsin activity was then stopped by adding 10 μl of 5% formic acid in 80% acetronitrile (ACN), and the digested peptides were dried by a SpeedVac concentrator (Savant; Holbrook, NY). The peptides were finally resuspended in 0.1% formic acid prior to MS/MS analysis.

2.6. Identification of proteins by nanoLC-ESI-Qq-TOF MS/MS

Separation of the digested peptides was performed using EASY-nLC II (Bruker Daltonics; Bremen, Germany). Briefly, peptides were loaded from a cooled (7 °C) autosampler into an in-house, 3-cm-long precolumn containing 5-μm C18 resin (Dr. Maisch GmbH; Ammerbuch, Germany) and then to an in-house, 10-cm-long analytical column packed with 3-μm C18 resin (Dr. Maisch GmbH) using mobile phase A (0.1% formic acid). The peptides were then separated by mobile phase B (ACN/0.1% formic acid) gradient elution (3–35%) for 150 min at a flow rate of 300 nl/min. Peptide sequences were then analyzed by an ultra-high resolution Qq-TOF MS/MS system (maXis Impact, Bruker Daltonics) in positive mode with ESI nanosprayer ion source. The nanoLC and Qq-TOF MS/MS systems were controlled by HyStar version 3.2 (Bruker Daltonics) and otofControl version 4.1 (Bruker Daltonics), respectively. A capillary voltage and spray shield voltage were set at 5000 V and 500 V, respectively. Nebulizer gas was set at 5.0 psi and dry gas flow rate was at 4.0 l/min, 150 °C [15].

For MS scanning, precursor ions were scanned from 50 to 2200 *m/z* range (resolution = 40,000 at 622 *m/z*) and acquired at 2 Hz (0.5 s total accumulation). For MS/MS experiment, the three most intense precursor ions for every MS scan were selected for further fragmentation. Collision-induced dissociation (CID) MS/MS acquisition was performed at 2 Hz (0.5 s total accumulation, if precursor $\leq 1 \times 10^4$ ion counts) and 10 Hz (0.1 s total accumulation, if precursor $\geq 5 \times 10^5$ ion counts) on the same mass range and resolution set for MS scanning, whereas singly charged ions were excluded. Smart exclusion parameters were set to minimize repeated acquisitions of the same intense precursor ions (repeated count was 2, dynamic exclusion was set at 0.50 min).

2.7. MS/MS data processing and protein quantitative analysis

The raw files (.d) were charge-deconvoluted and extracted into peak list files (.mgf) using Data Analysis version 4.1 software (Bruker Daltonics) via an embedded daMGF script. For identification, the peak