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Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice

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ABSTRACT

Several studies have characterized exosomes derived from different cell sources. In this work we set the goal of proteomic characterization of two less studied populations of membrane vesicles, microvesicles (100–800 nm) and apoptotic bodies (>800 nm) released by thymus cells of BALB/c mice. The vesicles were isolated by the combination of differential centrifugation and gravity driven multistep filtration of the supernatant of thymus cell cultures. The size distribution of vesicle preparations was determined by transmission electron microscopy. Proteins were released from the vesicles, digested in solution, and analyzed using nano-HPLC/MS(MS). Ingenuity pathway analysis was used to identify functions related to membrane vesicle proteins. In apoptotic bodies and microvesicles we have identified 142 and 195 proteins, respectively. A striking overlap was detected between the proteomic compositions of the two subcellular structures as 108 proteins were detected in both preparations. Identified proteins included autoantigens implicated in human autoimmune diseases, key regulators of T-cell activation, molecules involved in known immune functions or in leukocyte rolling and transendothelial transmigration. The presence and abundance of proteins with high immunological relevance within thymocyte-derived apoptotic bodies and microvesicles raise the possibility that these subcellular structures may substantially modulate T-cell maturation processes within the thymus.

1. Introduction

Thymus is a central immune organ that provides an environment for positive and negative selections of T lymphocyte maturation. It is the site of central tolerance induction and generation and shaping of the self-tolerant T-cell repertoire. Thus, it substantially contributes to protective cellular immunity and prevents pathological autoimmunity.

Thymus is distinguished from other tissues by two striking features. First, it is characterized by an extremely high apoptosis rate of thymocytes: it is estimated that approximately 95% of T-cell precursors die by apoptosis during thymic development [1]. The other unique feature is the ectopic gene expression of a wide range of tissue-specific antigens. Stromal medullary thymic epithelial cells (MTECs) express thousands of genes whose expression was earlier considered to be

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restricted to parenchymal organs [2]. Since the estimated number of MTECs is very low, the possible existence of intercellular antigenic transfer mechanisms (such as membrane vesicular transfer), has been raised [3]. Such a mechanism could efficiently disseminate tissue specific antigens to a high number of thymic antigen presenting cells, and would add another important function to the unexpectedly long list of immune processes in which the role of membrane vesicles has been documented [4].

Extracellular membrane vesicle is the collective term of nm size, subcellular structures originating from different cell types including both prokaryotic and eukaryotic cells (as we reviewed recently [5]). Their formation has been shown to be strongly enhanced upon activating or apoptotic stimuli. They are surrounded by a phospholipid bilayer, and enclose different molecular components (e.g. proteins, mRNA, miRNA) of the donor cell [6]. The best characterized membrane vesicle populations include microvesicles (in clinical studies often referred to as microparticles) with a diameter of 100–800 nm and exosomes that have a diameter <100 nm. Microvesicles (MVs) are formed by outward budding of the plasma membrane, while exosomes are released when the plasma membrane fuses with internal compartments of cells that contain intraluminal vesicles [7]. Surprisingly, apoptotic bodies (ABs) (with a diameter>800 nm), generated by similar mechanism as MVs, are often out of focus of studies of membrane vesicles, in spite of the fact that the principles of the isolation and detection of all subcellular membrane vesicles are similar. Recently, a rapidly increasing number of studies have characterized the exosomal protein composition by using mass spectrometry (for recent review see [8]). Data regarding the exosomal proteome are summarized in the Exocarta database ([http://exocarta.ludwig.edu.au/, [9]). Similarly, numerous works have characterized proteomic profiles of MVs, in particular those in human blood plasma [10–14].

Strikingly, until recently comparative mass spectrometry analysis of MVs and ABs has received little attention, and proteomic data for thymic vesicles are essentially lacking. Given the central role of thymus in immune cell development and immune tolerance induction, we focused on the proteomic characterization of thymocyte-derived ABs and MVs in order to gain an insight into the potential biological significance and functions of these subcellular structures.

2. Materials and methods

2.1. Cell culture

Thymuses of two weeks old BALB/c mice were removed, dissected into small pieces, and digested with 2 mg/mL collagenase D (Roche Applied Science, Indianapolis, IN, USA) in PBS for 30 min at 37 °C. Single cell suspension was made by repeated pipetting of the digested tissue, and was submitted to filtration through a 5 μm filter (Millipore) by gravity in order to remove cell aggregates. Cells were centrifuged at 300 *g* for 10 min, and the red blood cells were lysed in the pellet by addition of 10 mL of red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA in distilled H_20). After 60 s the cell suspension in lysis buffer was supplemented to a final volume of 50 mL. The non-lysed cells were pelleted, and 1.4×10^9 cells were transferred to a CELLine bioreactor (Integra Biosciences, Chur, Switzerland). Thymocytes were cultured in the lower compartment of the bioreactor in 15 mL DMEM supplemented with 10% membrane vesicle-free FCS (Gibco, NY, USA) and 2× concentration of glutamine (Sigma-Aldrich Co, St. Louis, MO, USA) and 4.5 g/L glucose. The upper compartment of the bioreactor contained 350 mL DMEM without FCS. After culturing for 24 h, the cells and their supernatant were collected from the lower compartment of the bioreactor. Cells were tested for viability using trypan blue exclusion test and for apoptosis by Annexin V-FITC (BD) binding using flow cytometry. In the population of thymocytes 30.92±4.08% were found positive for Annexin V binding after 24 h culture in CELLine bioreactor.

2.2. Isolation of microvesicles and apoptotic bodies

Fifteen mL supernatant from thymocyte cultures was pelleted at 300 *g* for 10 min. This was followed by filtration through a 5 μm pore size filter (Millipore Co. Cork, Ireland) by gravity (in order to avoid dispersion upon applying pressure by a syringe plunger).

ABs were pelleted at 2000 *g* for 20 min, followed by filtration through a 0.8 μm pore size filter (Millipore) by gravity. Next, the filtrate was centrifuged at 12200 *g* for 40 min to sediment MVs. The pellet was further washed two more times in PBS (by centrifugation at 12200 g for 10 min each time), and then re-suspended in 50 μL deionized water and stored at −80 °C. The presence of contaminating non-vesicular protein aggregates was excluded by 0.05% Triton X lysis of the membrane vesicle preparations as described previously [15].

2.3. Electron microscopy of membrane vesicles

Cellular and vesicular (AB and MV) pellets gained by combined differential centrifugation and filtration steps, were submitted for transmission electron microscopy. Briefly, after short centrifugation the supernatant was carefully removed, and the pellets were fixed at room temperature for 60 min. The fixative contained 2% paraformaldehyde and 2% glutharaldehyde in 0.01 M phosphate buffer, pH 7.4. After washing out the fixative with phosphate buffer, preparations were post-fixed in 1% OsO_4 (Taab; Aldermaston, Berks, UK) for 30 min. Following rinsing with distilled water, the pellets were dehydrated in graded ethanol, including block-staining with 2% uranyl acetate in 70% ethanol for 30 min, and embedded in Taab 812 (Taab). After overnight polymerization at 60 °C, ultrathin sections were analyzed with a HITACHI 7100 electron microscope. Electron micrographs were made by Megaview II (lower resolution, Soft Imaging System, Munster, Germany) digital camera. Brightness and contrast were adjusted when necessary by using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

2.4. Western blot analysis

Whole cell, MV and AB lysates were prepared using ProteoJet cell lysis buffer (Fermentas, Burlington, ON, Canada) supplemented