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A novel 3D heterotypic spheroid model for studying extracellular vesicle-mediated tumour and immune cell communication

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ABSTRACT

Cancer-derived extracellular vesicles (EVs) have emerged as important mediators of tumour-host interactions, and they have been shown to exert various functional effects in immune cells. In most of the studies on human immune cells, EVs have been isolated from cancer cell culture medium or patients' body fluids and added to the immune cell cultures. In such a setting, the physiological relevance of the chosen EV concentration is unknown and the EV isolation method and the timing of EV administration may bias the results. In the current study we aimed to develop an experimental cell culture model to study EV-mediated effects in human T and B cells at conditions mimicking the tumour microenvironment. We constructed a human prostate cancer cell line PC3 producing GFP-tagged EVs (PC3-CD63-GFP cells) and developed a 3D heterotypic spheroid model composed of PC3-CD63-GFP cells and human peripheral blood mononuclear cells (PBMCs). The transfer of GFP-tagged EVs from PC3-CD63-GFP cells to the lymphocytes was analysed by flow cytometry and fluorescence imaging. The endocytic pathway was investigated using three endocytosis inhibitors. Our results showed that GFP-tagged EVs interacted with a large fraction of B cells, however, the majority of EVs were not internalised by B cells but rather remained bound at the cell surface. T cell subsets differed in their ability to interact with the EVs - 15.7–24.1% of the total $CD3^+$ T cell population interacted with GFP-tagged EVs, while only 0.3–5.8% of $CD8^+$ T were GFP positive. Furthermore, a fraction of EVs were internalised in $CD3^+$ T cells via macropinocytosis. Taken together, the heterotypic PC3-CD63-GFP and PBMC spheroid model provides the opportunity to study the interactions and functional effects of cancer-derived EVs in human immune cells at conditions mimicking the tumour microenvironment.

1. Introduction

Extracellular vesicles (EVs), a heterogeneous group of nanosized membrane vesicles comprising exosomes, microvesicles and apoptotic bodies, have emerged as important mediators of intercellular communication and been implicated both in normal physiological processes and in the development of various diseases [1]. Cancer-derived EVs have been found in all body fluids and shown to transfer diverse signalling molecules, such as proteins, lipids, various coding and non-coding RNAs and even DNA fragments, from the cancer cells to various recipient cells, including immune cells, thus re-programming their functions [2,3].

Human cancer-derived EVs have been shown to induce T cell apoptosis and impair $CD8^+$ T cell cytotoxic activity [4–7], modulate Treg suppressor functions [8–11], activate cancer associated macrophages [12,13], and stimulate B cell proliferation and differentiation toward a plasmablast-like phenotype *in vitro* [14]. In all of these studies, EVs were isolated from cancer cell culture medium or patients' body fluids and added to the immune cell cultures. The effects have been shown to be EV concentration dependent [5,7,8]. Importantly, the assay readouts could be affected by the EV isolation method, purity of EVs as well as timing of the EV administration. In most of the studies, EVs were quantified by measuring the

Abbreviations: EVs, extracellular vesicles; PBMCs, peripheral blood mononuclear cells; HD, healthy donor; GFP, green fluorescent protein; FACS, fluorescence activated cell sorting; MVs, microvesicles; TME, tumour microenvironment.

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total EV protein concentration and the EV amounts per cell ranged from 0.04 to 4 ng of EV proteins in the functional assays [4–7]. The physiological relevance of such EV concentrations, however, remains unknown.

In this study, we have constructed a human prostate cancer cell line PC3 producing GFP-tagged EVs (PC3-CD63-GFP cells) and developed a three-dimensional (3D) heterotypic spheroid model composed of PC3-CD63-GFP cells and human peripheral blood mononuclear cells (PBMCs). Our data show that this model can be used for the tracking and quantification of EV uptake in various PBMC populations. Moreover, the model could be exploited for studying the mechanisms of interaction and functional effects of cancer-derived EVs in various tumour stroma cell subsets.

2. Materials and methods

2.1. Cell culture and transfection

The human prostate cancer epithelial cell line PC3 was obtained from ATCC (Manassas, VA, USA). PC3 cells were cultured in Ham's F-12/DMEM (1:1 mixture) with Glutamax (Invitrogen, USA), supplemented with 7% foetal calf serum (FCS) (Sigma-Aldrich, USA), 100 units/ml penicillin and 100 units/ml streptomycin (Sigma-Aldrich, USA), in a humidified 5% CO_2 atmosphere at 37 °C.

PC3-CD63-GFP cells were generated by transfecting PC3 cells with a pEGFP-C1 vector (Clontech, USA) encoding for GFP fusion protein with human CD63 under CMV promoter. Cells (1.5×10^6) were seeded in 100 mm plates 24 h before transfection. Transfection was performed with FuGENE® 6 transfection reagent according to the manufacturer's protocols (Promega, USA). The day after transfection, cells were detached, counted and resuspended at the concentration of 2×10^6 cells/ml in medium containing serum and antibiotics and transferred into a sorting tube. GFP expressing cells were sorted using FACS Aria II SORP high speed sorter into a 15 ml tube containing 3 ml complete medium. Sorted cells were pelleted for 5 min at 300 g and maintained in complete medium containing 0.5 mg/ml Geneticin (Thermo Fisher Scientific, USA). Two weeks after the first sorting, GFP expressing cells were sorted again using the same protocol as before, and maintained in complete medium containing 0.1 mg/ml Geneticin. At this point, PC3-CD63-GFP cells were characterised by immunoblotting and immunocytochemistry analysis.

2.2. EV isolation

EVs were isolated from the PC3-CD63-GFP cell-conditioned medium after 18–19 h incubation as previously described [15]. Briefly, the medium was centrifuged at 1000 g for 10 min to remove dead cells and cell debris, and thereafter at 10,000 g for 30 min to pellet the microvesicle (MV) fraction. The supernatant was then ultracentrifuged at 100,000 g for 70 min. The exosome-enriched EV pellet was washed with PBS, and centrifuged again at 100,000 g for 70 min. All centrifugation steps were carried out at 4 °C.

2.3. SDS-PAGE and western blot

Cell lysates were prepared by washing cells twice with cold PBS before adding lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.4) containing a protease inhibitor cocktail (Roche Applied Science, Germany). The cells were lysed on ice for 20 min and then centrifuged at 20,000 g for 10 min at 4 °C. EV pellets were resuspended in lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.2% SDS lauryl, pH 7.4). EV lysates and cell lysates were mixed with loading buffer, heated at 95 °C for 5 min and loaded on 4–20% gradient TGX gels (Bio-Rad, USA). After SDS-PAGE the proteins were transferred to PVDF membranes (Whatman, Germany) using a Transfer-Blot Turbo Transfer Pack (Bio-Rad, USA). Membranes were incubated with the mouse anti-CD63 (H5C6 clone, DSHB, USA) and rabbit anti-GFP (Santa Cruz Biotechnology, USA) primary and respective HRP-conjugated secondary antibodies (Jackson Immunoresearch, USA). Blots were visualized with the Pierce SuperSignal West Dura Extended Duration (Thermo Scientific, USA) on the Universal Hood II Bio-Rad scanner (Bio-Rad, USA).

2.4. Generation of 3D heterotypic spheroid culture

PBMCs were obtained from four healthy male volunteers using BD Vacutainer CPT™ Cell Preparation Tube with Sodium Heparin (BD Biosciences, USA). PBMCs were washed twice with PBS, counted and immediately used for experiments. The study was approved by the Ethics Committee of Institute of Experimental and Clinical Medicine, University of Latvia, and the volunteers were enrolled after an informed consent was obtained.

To generate 3D heterotypic spheroid cultures, PBMCs were mixed with PC3-CD63-GFP cells at a ratio of 1:1 and cultured in serum free DMEM-F12 medium (Lonza, Switzerland) supplemented with 1 × B27 (Thermo Fisher Scientific, USA), 2 mM L-glutamine, 1 × antibiotic-antimycotic (Thermo Fisher Scientific, USA), 20 ng/ml hEGF (RnD Systems, USA), 10 ng/ml basic hFGF (Santa Cruz Biotechnology, USA) and 0.5 μg/ml hydrocortisone (Sigma-Aldrich, USA). To ensure the formation of heterotypic spheroids, cells were seeded at high density (5×10^5 PBMCs and 5×10^5 PC3-CD63-GFP cells per 1.5 ml of medium) in 6-well flat bottom suspension plates (Sarstedt, Germany) and cultured for 72 h at 37 °C with 5% CO_2 in a humidified incubator (Panasonic, Panasonic Healthcare Co., Ltd.).

2.5. Treatment with endocytosis inhibitors

The spheroids were cultured with or without the following endocytosis inhibitors: ethylisopropyl amiloride (EIPA) (Cayman Chemical, USA), nystatin and dynasore (Sigma-Aldrich, USA) (all 20 μM) for 72 h. The cytotoxicity of the compounds in PBMCs and PC3 cells was tested prior to analysis using CCK-8 assay (Sigma-Aldrich, USA) according to manufacturer's instructions.

2.6. Flow cytometry

After 72 h of co-culturing, the 3D PBMC - PC3-CD63-GFP spheroids were collected, disintegrated using Accutase solution (Sigma Aldrich, USA) and washed with PBS. Cells were stained with anti-CD3-PerCP eFluor 710 (clone SK7), anti-CD8-eFluor 450 (clone SJ25C1) (both from eBioscience, Thermo Fisher Scientific, USA) or anti-CD19-PE (Santa Cruz Biotechnology, USA) antibodies for 1 h at room temperature in the dark and analysed with the BD FACSAriaII instrument. The fluorophore compensation was calculated using cells with single staining, and the control and auto fluorescence percentage was subtracted from the results.

2.7. Immunofluorescence

For visualisation of GFP-CD63, PC3 cells grown on glass coverslips were fixed in 4% paraformaldehyde and mounted with ProLong Gold antifade mounting medium with DAPI (Molecular Probes, USA). The cells were imaged using a Zeiss LSM780 laser scanning confocal microscope (Carl Zeiss MicroImaging, Germany) equipped with an Ar-Laser multiline (458/488/514 nm), a DPSS-561 10 (561 nm), and a Laser diode 405–30 CW (405 nm). The objective used was a Zeiss Plan-Apochromat 63 × /1.40 Oil DIC M27. Images

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