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Original Articles

Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant B-lymphocytes

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ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive and incurable mature B cell neoplasm. The current treatments are based on chemotherapeutics and new class of drugs (e.g. *Ibrutinib*®), which in most cases ends with tumor resistance and relapse. Therefore, further development of novel therapeutic modalities is needed. Exosomes are natural extracellular vesicles, which play an important role in intercellular communication. The specificity of exosome uptake by different target cells remains unknown. In this study, we observed that MCL exosomes are taken up rapidly and preferentially by MCL cells. Only a minor fraction of exosomes was internalized into T-cell leukemia and bone marrow stroma cell lines, when these cells were co-cultured with MCL cells. Moreover, MCL patients' exosomes were taken up by both healthy and patients' B-lymphocytes with no apparent internalization to T lymphocytes and NK cells. Exosome internalization was not inhibited by specific siRNA against caveolin1 and clathrin but was found to be mediated by a cholesterol-dependent pathway. These findings demonstrate natural specificity of exosomes to B-lymphocytes and ultimately might be used for therapeutic intervention in B cells malignancies.

Introduction

Mantle cell lymphoma (MCL) is a well-defined, aggressive, lymphoid neoplasm characterized by proliferation of a distinctive population of mature B-lymphocytes [1]. The neoplastic cells tend to colonize the mantle zone of the lymphoid follicles and spread throughout the body, infiltrating lymphoid tissues, bone marrow, peripheral blood and extranodal sites [2]. Conventional chemotherapy induces remission in the majority of newly treated patients. However, within a few years, most patients experience relapse with severe drug-resistant phenotype that often leads to death with relatively short median survival duration of 5–7 years [3]. Therefore, the discoveries of novel biological pathways involved in MCL progression and resistance may lead to new therapeutic agents with improved treatment outcomes [4].

Exosomes are small extracellular membrane-enclosed vesicles of 30–150 nm in diameter. Exosomes originate from the inward budding of the endosomal membrane, forming multivesicular bodies (MVBs). They are secreted into the extracellular environment or into biological fluids as a result of fusion of intracellular MVBs with the plasma membrane and are considered as messengers in intercellular communication [5,6]. Exosomes are released by different types of healthy cells such as leukocytes (B lymphocytes [7,8], T lymphocytes [9] and natural killer cells (NK) [10]) as well as by cancer cells, lymphocytic cell lines [10,11] and by primary chronic lymphocytic leukemia (CLL) and acute myelogenous leukemia (AML) cells [12,13]. Their biological role depends on the cell of origin and recipient cell. The putative function of exosomes in cancer is based on the recently described findings of transfer of genetic material and signaling proteins, resulting in increased angiogenesis, metastasis, drug resistance and immunosuppressive environment [5,14–16]. Increasing evidence emphasize the role of exosomes secreted by various leukemic cells in reprograming the microenvironment and supports disease progression. Exosomes released by K562 cells, a chronic myeloid leukemia (CML) cell line induce angiogenic activity of human umbilical endothelial cells (HUVEC) [17]. These exosomes when released from K562 cells overexpress microRNA-92a, were able to transfer this microRNA to HUVEC, and enhance their migration and blood vessel formation [18]. Recently, CML-derived exosomes were shown to promote, through an autocrine mechanism, the proliferation and survival of tumor cells, both *in vitro* and *in vivo*, by activating anti-apoptotic pathways [19]. Primary and cell line AML-derived exosomes

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transfer mRNA and alter the secretion of growth factors from bone marrow stromal cells, thereby supporting the survival of the neoplastic cells [13]. Furthermore, leukemia and lymphoma derived exosomes were shown to immunosuppress the cytotoxic activity of NK cells by binding the NK group 2 receptor (NKG2D), thus assisting in the immune evasion of malignant cells [20].

Both endocytosis [21–23] and phagocytosis [24] pathways involved in delivery of functional cargo to recipient cells [25,26]. Proteomic analysis of exosomes reveals elevated levels of adhesion proteins that promote absorption to the cell surface [27,28]. In addition, exosomes have been shown to attach to recipient cells via phosphatidylserine receptors [29]. Previous studies have suggested that tumor derived exosomes are more readily associated with cancer cells as compared to normal cells ex-vivo [30,31]. However, specificity of exosomes to recipient cells and the membrane molecules that are involved in the recognition of exosomes by recipient cells are mostly unknown. In this study, we characterized exosomes of the MCL cell line and primary cells, elucidate their internalization mechanism and decipher their cell specificity.

Materials and methods

Simvastatin, Dynasore, PKH-26 and PKH-67 were purchased from Sigma-Aldrich, Israel. Nystatin was purchased from Biological Industries Ltd, Israel. Gefitinib was pursued from Cayman Chemicals, USA. All other materials were of chemical grade. MEBCYTO® apoptosis kit was purchased from MBLI.

siRNA sequences

siCLTC and siCAV1 were purchased from siGENOME SMARTpool – Thermo Scientific Dharmacon. The following combination of four different siRNA oligos for clathrin heavy chain (CLTC) and caveolin1 (CAV1) were employed:

siCLTC – (GCAAUGAGCUGUUUGAAGA; GAAAGAAUCUGUAGAGAAA; UGACAAAGGUGGAUAAAUU; GGAAAUGGAUCUCUUUGAA).
siCAV1 – (GCAAAUACGUAGACUCGGA; AUUAAGAGCUUCCUGAUUG; GCAGUUGUACCAUGCAUUA; CUAAACACCUCAACGAUGA). The luciferase gene as control sequence (siLUCsense strand: CUUACGCUGAGUACUUCGA) were designed and screened by Alnylam Pharmaceuticals (Cambridge, MA, USA).

Cell culture

Mantle cell lymphoma cell line, Jeko-1 (CRL-3006) was purchased from Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) and Mino cell line (CRL-3000) was purchased from the American Type Culture Collection (ATCC). Jeko-1 and Mino cell lines were cultured in RPMI-1640 (Gibco, Life Technologies), supplemented with 20% or 15% exosome-depleted, Fetal Bovine Serum (FBS) (Biological Industries Ltd, Israel), respectively supplemented with 2% glutamine (Gibco, Life Technologies) and 1% penicillin/streptomycin (Biological Industries Ltd, Israel). Jurkat (TIB-152), human acute T cell leukemia cell line and HS-5 (CRL-11882) human bone marrow derived stroma cell line were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies), supplemented with 10% exosome-depleted FBS (Biological Industries Ltd, Israel), 2% glutamine (Gibco, Life Technologies) and 1% penicillin/streptomycin (Biological Industries Ltd, Israel).

Healthy and MCL patients' mononuclear cell isolation

Peripheral blood (PB) and serum samples were obtained from healthy donors and from patients with MCL who were treated at the Rabin Medical Center (Petah Tikva, Israel) and the Rambam Medical Center (Haifa, Israel) after obtaining institutional review board-approved informed consent. The clinical characteristics of the patients whose PB samples were studied are presented in Table 1. To isolate low-density cells, PB cells were fractionated using Ficoll-Paque™ PLUS (GE Healthcare, Life Sciences). Fractionated cells were used immediately or frozen for additional studies.

Exosome isolation and labeling

Exosomes were isolated by differential centrifugation [32] and were labeled by PKH-26 red/PKH-67 green fluorescent cell linker cell membrane labeling (Sigma-Aldrich) as described in Supplemental Materials and Methods. Exosomes were isolated from serum by total exosome isolation reagent from serum (Life Technologies).

Exosome analysis by flow cytometry

Anti CD81 coated latex beads were bound to exosomes and analyzed by flow cytometry as described in Supplemental Materials and Methods.

Structural analysis of exosomes by electron microscopy

MCL Exosomes were loaded onto formvar carbon coated grids (Ted Pella Inc, Redding, USA). Next, the exosomes were fixed in 2% paraformaldehyde and washed. The exosomes were immunostained with anti-CD81 antibody (BioLegend) followed by staining with a 12 nm gold-conjugated secondary antibody (Jackson ImmunoResearch). Staining with 12 nm gold-conjugated secondary antibody only was used as a negative control. The exosomes were subsequently fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate and embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%). The preparations were examined with Jeol 1200EX TEM (Jeol, Japan).

Nanoparticle tracking analysis (NTA)

Size distribution analysis of exosomes based on Brownian motion was assayed by NanoSight LM20 (NanoSight, Amesbury, United Kingdom) using NTA2.3 software upon exosomes dilution into PBS pH 7.4.

Exosomes internalization assays

Exosomes were labeled with PKH-26 for confocal microscopy experiments or with PKH-67 for flow cytometry analysis experiments, and uptake was determined by flow cytometry and confocal microscopy as described in Supplemental Materials and Methods.

Western blot analysis

Lysates of cells and exosomes were separated by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with antibodies as described in Supplemental Materials and Methods.

Electroporation

1 nmole of each of the RNA duplexes (siCLTC, siCAV or siLUC) was electroporated into 10×10^6 Jeko-1 cells using the Amaxa 4D-nucleofactor system (CM-119 program, SF cell line solution).

Table 1
MCL patient characteristics.

MCL patient no.	PB/BM	Sex	Age Y	WBCs $10^3/\mu l$	Lymphocytes (%)	Hemoglobin g/dl	Platelets $10^3/\mu l$	Immuno-histochemistry cyclin D1	IgH/CCND1 FISH	Alive = A Death = D	Previous treatment
1	PB	M	65	16.7	10	13.6	78	Positive	Positive	D	
2	PB	M	50	3.37	19	8.3	79	Positive	Positive	A	
3	BM	M	51	6.38	33	13.1	139	Positive	Positive in PB	A	RCHOP
4	PB	F	57	223	81	9.8	112	Positive	Positive	D	
5	PB	M	62	104	94	10.2	96	Positive	Positive	D	
6	PB	M	75	94.35	86	12.3	93	Positive	NA	A	
7	BM	M	65	15.56	10	9.3	33.8	Positive	Positive	A	
8	PB	M	75	94.35	92	12.3	93	Positive	positive	A	

MCL indicates mantle cell lymphoma; PB, peripheral blood; BM, bone marrow; M, male; F, female; WBC, white blood cell; CCND1, cyclin D1; NA, not available; RCOP, rituxan, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone.