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Original Article

Murine and human pancreatic tumor exosome recovery in mouse serum: Diagnostic and prognostic potential and target cell delivery

Ulrike Erb, Kun Zhao, Zhe Wang, Li Xiao, Margot Zöller*

Tumor Cell Biology, University Hospital of Surgery, Heidelberg, Germany

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ABSTRACT

Exosomes (Exo), powerful intercellular communicators, are recovered in all body fluids, suggesting suitability for diagnosis and prognosis. Easy in vitro manipulation recommends Exo as drug vehicles. Aiming to consolidate diagnostic and therapeutic potential of Exo, we evaluated recovery and fate of tumor (TEX) and exogenous Exo in syngeneic and xenogeneic mice bearing a murine or a human pancreatic adenocarcinoma.

A significant increase in serum (S)-TEX was observed 2 weeks after tumor cell application. Instead, S-TEX declined within 3–6 days after tumor excision. Intravenously injected dye-labeled TEX were rapidly cleared from the serum. Partly being degraded in the liver, the majority is taken-up by PBL, liver, bone marrow and lung cells. In the tumor-bearing host TEX persisted longer becoming enriched in tumor cells and metastatic organs. Accordingly, an antibody blockade of a TEX marker hampered disseminated tumor cell settlement in selected organs.

In brief, a tumor marker panel appears suited for S-TEX recovery. In murine models, S-TEX are qualified for therapy control and follow-up studies. Despite rapid clearance from the serum, Exo uptake by host cells is most promising for tailored Exo as drug transporter.

Introduction

Pancreatic adenocarcinoma (PaCa) frequently is diagnosed after metastatic spread or expansion of the local tumor prohibiting surgery, the only curative option [1,2]. Imaging being of limited help in early detection [3], great hope is given to serum-based diagnosis, being low-invasive and suited for follow-up studies. The standard serum marker CA19-9 displays too low sensitivity and specificity [4]. Though free serum miRNA recovery repeatedly showed promising results [5,6], lncRNA- and miRNA-based serum diagnosis frequently is burdened by abundant delivery from peripheral blood leukocytes (PBL) [7]. Tumor-derived vesicles, particularly tumor exosomes (TEX), which are enriched for selective components, offer an alternative [8].

Exosomes (Exo), small 40–130 nm vesicles delivered by many cells and abundantly by tumor cells [9], are found in all body fluids [10]. Exo biogenesis starts with the formation of early endosomes (EE), which can derive from the *trans*-Golgi network or from different internalized membrane microdomains, like clathrin-coated pits, tetraspanin and glycolipid-enriched membrane domains, or proteolipids in cholesterol- and ceramide-rich compartments [11,12]. EE move towards multivesicular bodies (MVB), the transport machinery varying for the different types of EE [13]. During inward budding of EE into MVB, called intraluminal vesicles (ILV), vesicles receive their cargo, proteins, coding and noncoding RNA and DNA in non-random processes [11,14–16]. MVB fuse with the plasma membrane, the released ILV are called Exo [11,14]. Due to the differences in biogenesis, single cells can deliver different Exo [17,18]. This is important for selecting potential diagnostic markers.

Exo research became highly stimulated, when it was noted that antigen-presenting cells release Exo, which can replace the donor

Abbreviations: Alix, ALG-2 interacting protein X; Ann, Annexin; BMC, bone marrow cells; CIC, cancer initiating cell; EE, early endosomes; EpC, EpCAM; ESCRT, endosomal sorting complex required for transport; Exo, exosomes; FCS, fetal calf serum; Fbg, fibrinogen; FI, fluorescence index; GEM, glycolipid-enriched membrane domains; Glp1, glypican1; HSP, heat shock protein; ILV, intraluminal vesicles; i.v., intravenous; LB, latex beads; LNC, lymph node cells; MVB, multivesicular bodies; o.t., orthotopic; PaCa, pancreatic adenocarcinoma; PBL, peripheral blood leukocytes; S, serum; s.c., subcutaneous; SC, spleen cells; Synd, Syndecan; TB, tumor-bearer; TEX, tumor exosomes; Tsg, tumor susceptibility gene; Thsp, thrombospondin.

* Corresponding author. Tumor Cell Biology, University Hospital of Surgery, Im Neuenheimer Feld 365, D-69120 Heidelberg, Germany.
E-mail address: m.zoeller@uni-hd.de (M. Zöller).

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cell [19]. The discovery that all Exo components are function competent and that Exo can transfer coding and non-coding RNA and DNA in host cells inducing exogenous gene expression and mediating RNA silencing [20,21] further spurred Exo research. A third boost followed, when it was suggested that Exo recovery in body fluids can serve for non-/minimally invasive diagnosis and therapy control [22,23].

Diagnostic validity was mostly controlled for TEX protein and miRNA markers [22]. Except few reports on high sensitivity and specificity of a single marker [24], marker panels were found to be more reliable in most instances [25–28], which is fostered by the heterogeneity of tumors and the delivery of different types of Exo by single cells [29]. Though deep sequencing likely provides an unequivocal answer to optimal marker panels, there remain two questions, which we started to approach.

The first question relates to the required quantity of Exo to guarantee reliable recovery in body fluids. The answer will vary with the location of the tumor and the evaluated body fluid. Nonetheless, for cancer in situ and grade I malignancies the question awaits an answer. Information on “free” TEX versus TEX bound to or uptaken by extracellular matrix, non-transformed cells or tumor cells also is missing. Our second question is concerned on the in vivo survival and distribution of Exo, the answer being particularly important for therapeutic Exo application, but the diagnostic potential of TEX also depends on the persistence in body fluids.

Material and methods

Cell lines

UNKC6141, a cell line deriving from a PaCa spontaneously arisen in a Kras(G12D); Pdx1-Cre mouse [30], were maintained in ISCOVE'sMEM/10%FCS/L-glutamine/antibiotics. The human A818.4 PaCa line [31] is maintained in RPMI1640/10%FCS/pyruvate/L-glutamine/antibiotics.

Antibodies

See Suppl. Table S1.

Tissue preparation and cell isolation

Mice were sacrificed by cervical dislocation. Single cell suspensions of draining lymph nodes (LNC), spleen (SC), liver, lung, kidney, and pancreas were prepared by pressing through fine gauze. BM cells (BMC) were collected by flushing femora and tibiae with PBS. Peripheral blood (PB) was collected by heart puncture, syringes containing 100U heparin. PBL were collected after FicollHypaque centrifugation.

TEX and serum Exo (S-Exo) preparation

Tumor cells were cultured (48 h) in serum-free medium. Cleared supernatants (2 × 10 min, 500 g, 1 × 20 min, 2000 g, 1 × 30 min, 10000 g) were centrifuged (120 min, 100000 g), the pellet was washed (PBS, 120 min, 100000 g), resuspended in 40% sucrose overlaid by a discontinuous sucrose gradient (30%–5%) and centrifuged (16 h, 100000 g). TEX were collected from the 10%–5% sucrose interface (light density fractions, d: 1.15–1.56 g/ml). S-Exo were purified from the plasma accordingly after dilution (1:5) in PBS. Where indicated, TEX/S-Exo were labeled with SP-Dio$_{18}$(3). After quenching (15 ml Exo-depleted FCS) and washing (2 × 120 min, 100000 g) TEX/S-Exo were suspended in 30 ml PBS layered over 10 ml 40% sucrose and centrifuged (120 min, 100000 g), collecting the Exo pellet at the bottom [32].

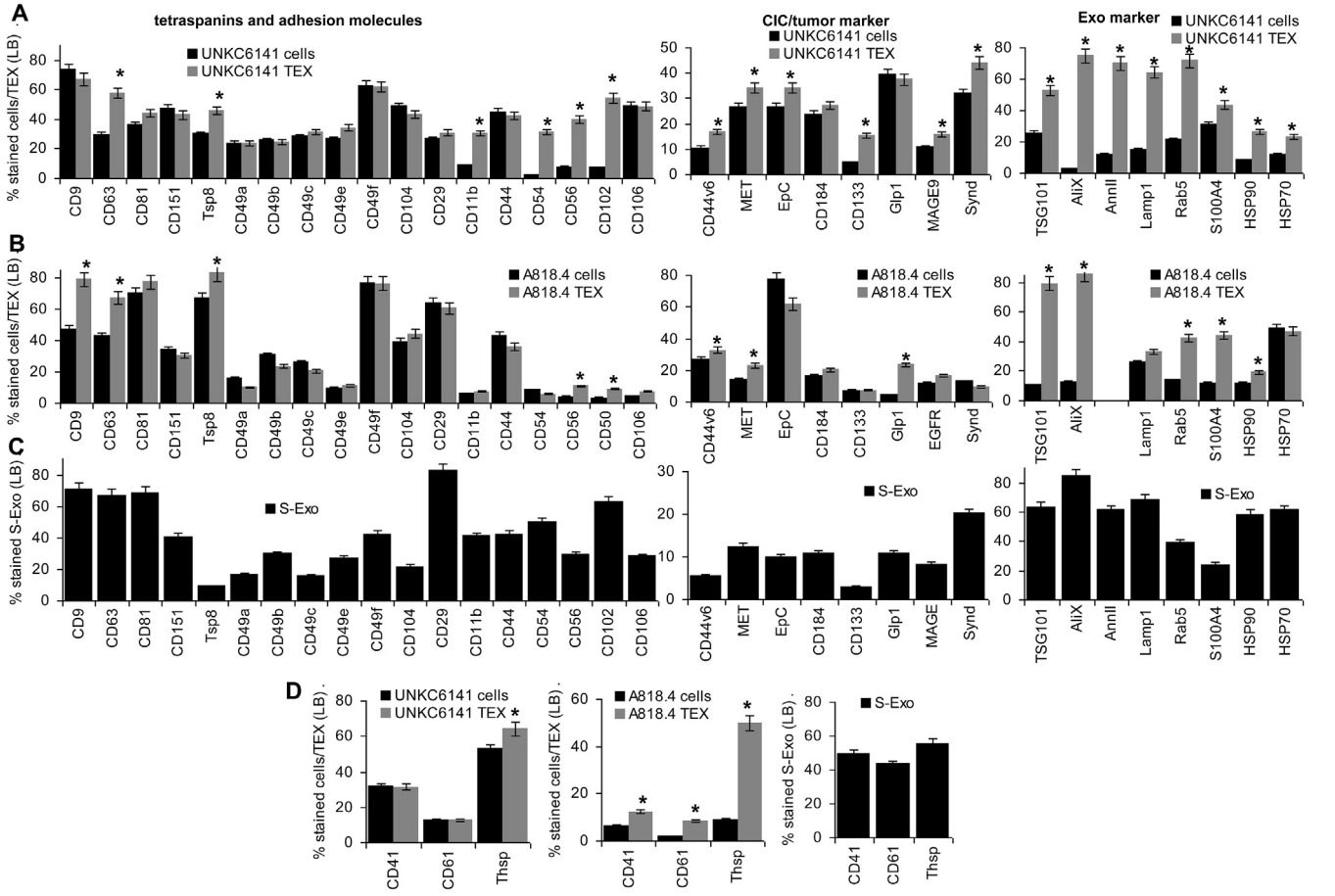

Fig. 1. Characterization of TEX and S-Exo. (A–D) TEX were collected from culture supernatant of UNKC6141 and A818.4 cells, S-Exo were collected from the plasma of healthy C57BL6 mice. Cells, TEX and S-Exo were characterized for expression of tetraspanins, adhesion molecules, CIC, Exo and tumor markers and platelet-derived Exo markers by flow-cytometry. The percent of stained cells and Exo-loaded LB (mean ± SD of 5 experiments) is shown; (A, B, D) significant differences between cells and TEX: *. Common Exo markers, including tetraspanins are highly expressed in TEX and S-Exo. CIC markers are mostly upregulated in TEX compared to cells and are recovered at a very low level on S-Exo. Adhesion molecule expression varies between the two tumor lines, only some adhesion molecules are upregulated in TEX. According to the expression profile in TEX, thrombocyte Exo markers are not suited for differential diagnosis.