Thrombosis Research 159 (2017) 24–32

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Breast-cancer extracellular vesicles induce platelet activation and aggregation by tissue factor-independent and -dependent mechanisms

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ARTICLE INFO

Keywords:
Thrombosis
Cancer
Extracellular vesicles
Exosomes
Microvesicles
Platelets

ABSTRACT

Introduction: Cancer-associated thrombosis is one of the major causes of worse prognosis among tumor-bearing patients. Extracellular vesicles derived from cancer cells, which can be divided mainly into microvesicles and exosomes, can participate in several tumor progression phenomena. Tumor-derived microvesicles positive for tissue factor (TF) have been associated with thrombotic risk in certain cancer types. Cancer cell-derived exosomes, however, have not. In this study we evaluated the capacity of extracellular vesicles (EVs, containing both microvesicles and exosomes) derived from breast-cancer cell lines in promoting platelet activation, aggregation and plasma coagulation, in experiments that access both TF-dependent and -independent activities.

Materials and methods: EVs were isolated from the conditioned media of two human mammary carcinoma cell lines: MDA-MB-231 (highly invasive) and MCF-7 (less invasive). TF-independent EV/platelet interaction, platelet P-selectin exposure and aggregation were evaluated. Western blotting, plasma clotting and platelet aggregation in the presence of plasma were performed for the measurement of TF-dependent activity in EVs.

Results: Interaction between MDA-MB-231 EVs and washed platelets led to increased platelet P-selectin exposure and platelet aggregation compared to MCF-7 EVs. MDA-MB-231 EVs had higher TF protein levels and TF-dependent procoagulant activity than MCF-7 EVs. Consequently, TF-dependent platelet aggregation was also induced by MDA-MB-231 EVs, but not by MCF-7 EVs.

Conclusion: Our results suggest that MDA-MB-231 EVs induce TF-independent platelet activation and aggregation, as well as TF-dependent plasma clotting and platelet aggregation by means of thrombin generation. In this context, aggressive breast cancer-derived EVs may contribute to cancer-associated thrombosis.

1. Introduction

Since the 19th century, it is known that cancer and thrombosis can be associated with each other [1–3]. Compared to the general population, cancer patients have a 4- to 7-fold greater chance of developing thromboembolic disease [4,5]. When a patient has cancer and a thromboembolic condition, the prognosis becomes much poorer, with increased mortality odds in comparison to patients with only cancer or thrombosis [6].

It is widely recognized that the clotting-initiating protein, tissue factor (TF), is expressed in several tumor types and is positively associated with advanced stages of the disease [7,8,9]. Tumor-derived TF has been associated with several pro-tumoral responses including primary growth, metastasis, angiogenesis and cancer-associated thrombosis [10,11,12]. In addition to the procoagulant properties conferred by TF, tumor cells can promote platelet activation and aggregation by TF-independent mechanisms [13,14,15]. Therefore, tumor-derived factors may support cancer prothrombotic phenotype by diverse

Abbreviations: EV, extracellular vesicle; TF, tissue factor; ACD, acid-citrate-dextrose; FITC, fluorescein isothiocyanate; PE, phycoerythrin; RIPA, radioimmunoprecipitation assay buffer

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http://dx.doi.org/10.1016/j.thromres.2017.09.019
Received 29 May 2017; Received in revised form 15 September 2017; Accepted 19 September 2017
Available online 21 September 2017
0049-3848/

mechanisms of action.

Extracellular vesicles (EVs) shed from cancer cells are often found in the bloodstream [16]. Such vesicles carry genetic material derived from cancer cells (such as mRNAs and siRNAs) as well as other tumor-derived biological molecules such as proteins and glycoconjugates that may systemically affect the host [17]. The two most-studied populations of extracellular vesicles shed by cancer cells are microvesicles (also called microparticles or ectosomes) and exosomes. Microvesicles are formed by shedding of cellular membrane portions, mainly in lipid-raft regions. It is a calcium-dependent process that involves loss of membrane bilayer assymmetry, phosphatidylserine exposure, loss of membrane anchoring to the cytoskeleton and microvesicle liberation [17,18]. Exosome formation begins with the invagination of membrane fragments, followed by formation of multivesicular bodies inside the cell, which can be directed to degradation and recycling or fuse with the cell membrane, liberating the exosomes by exocytosis [19]. Exosomes can interact with recipient cells in different ways, for example, through protein interactions, membrane fusion or endocytosis by the recipient cell [20]. It is generally accepted that microvesicles may range in sizes from 100 to 1000 nm, whereas exosomes from 30 to 100 nm [20].

A potential role for tumor-derived microvesicles in the establishment of cancer-associated thrombosis has long been proposed [12,21,22]. A number of studies have correlated increased plasma levels of tumor-derived tissue factor (TF)-positive microvesicles with a prothrombotic state in both animal models [23,24,25] and patients [26,27,28]. However, this correlation has not been observed in several cancer types including gastric, colorectal, brain and breast cancer [29,30]. Interestingly, Bang and collaborators have recently shown that overall levels of cancer cell-derived microvesicles in plasma of tumor-bearing patients were positively associated with thrombosis, but no relation was found between circulating TF-positive microvesicles and cancer-associated thrombosis [31].

Exosomes can participate in several tumor-associated processes, including angiogenesis, matrix degradation, pre-metastatic niche establishment, immunosuppression and drug resistance [32]. There is still no compelling evidence that tumor exosomes can directly influence cancer-associated thrombosis in humans. There are, however, published data that support a pro-hemostatic hypothesis of tumor exosomes. For example, patients with advanced-stage ovarian cancer have higher risk of developing thrombosis [33], and the concentration of cancer exosomes is higher in the circulation of patients that have advanced-stage ovarian cancer compared to early-stage patients, patients with benign tumors, or control subjects [34]. Furthermore, Svensson and colleagues have shown that glioblastoma exosomes have high TF protein levels [35].

Therefore, we hypothesize that EVs derived from cancer-cell lines may trigger pro-hemostatic responses by different pathways. The purpose of our work was to study a potential role of cancer EVs in pro-hemostatic events such as platelet activation, aggregation and plasma clotting; accessing both TF-dependent and -independent activities.

2. Materials and methods

2.1. Cell culture

The human breast-cancer cell lines MDA-MB-231 (highly invasive) and MCF-7 (less invasive) were maintained at 37 °C, 5% CO_2, grown in Iscove's modified Dulbecco's (LGC Bio, SP, Brazil) medium containing 1% penicillin-streptomycin (Gibco, NY, USA) and 10% fetal bovine serum (FBS; Cultilab, SP, Brazil), at pH 7.2.

2.2. Extracellular vesicle (EV) isolation

EVs were isolated using the precipitation solution ExoQuick-TC™ (System Biosciences, CA, USA) according to the manufacturer's instructions. Initially, conditioned medium was centrifuged at 3000 × *g*, 15 min, for removal of cell debris. ExoQuick-TC™ was then added to the conditioned media at a proportion of 1:5 (v/v) and left overnight at 4 °C. After the incubation, the samples were centrifuged at 1500 × *g* for 30 min. The supernatant was discarded and the pellet was washed at 1500 × *g*, for 5 min. Remnants of supernatant were aspirated with a pipette and the EV pellet was resuspended in Tris-buffered saline (TBS) for plasma-clotting experiments, RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium desoxycholate, 0.1% SDS) plus protease inhibitor (Complete Protease Inhibitor Tablets; Roche, Basel, Switzerland) for Western blotting and phosphate-buffered saline (PBS) for all other assays. EVs were isolated from serum-free media for the NTA, electron microscopy, CFSE-stained EV/platelet interaction and washed platelet aggregation experiments. EVs were isolated from 10% FBS-supplemented conditioned media for the remaining experiments.

For specific cases in which EVs were isolated by ultracentrifugation, cell supernatant was initially centrifuged at 3000 × *g* for 15 min to remove cell debris. The supernatant was then centrifuged at 20,000 × *g*, for 30 min, followed by ultracentrifugation at 100,000 × *g* for 120 min. The pellet was then resuspended in PBS.

EV concentration for the experiments was adjusted by protein concentration, which was determined by the Lowry method [36]. Reagents used were from the DC™ protein assay kit (Bio-Rad, CA, USA), and absorbance was read at 750 nm in a SpectraMax 190 microplate reader (Molecular Devices, CA, USA).

2.3. Nanoparticle tracking analysis (NTA)

NTA was performed on ZetaView® PMX 110 V3.0 (Particle Metrix GmbH, Meerbusch, Germany) to evaluate the size and concentration of EVs as previously described [37]. Briefly, EVs were diluted 1:20,000 in PBS (filtered cut-off 0.22 μm) and 1 mL of the sample was loaded into the equipment's cell. Two reading cycles were performed for each of the 11 positions analyzed. Acquisition parameters: Pre-acquisition - temperature 23 °C, sensitivity 85, frame rate 30 fps, shutter 55, and laser pulse duration equal to that of shutter duration. Post-acquisition - minimum brightness of 20, a maximum size of 200 pixels, and a minimum size of 5 pixels. The equipment calibration was performed with polystyrene beads (100 nm). In this step, were verified: cell quality, alignment, focus, temperature, conductivity, electrical field, and drift measurements. Data was analyzed with ZetaView 8.02.31 software [38].

2.4. Electron microscopy

Two different methodologies were used to characterize the ultrastructure of the extracellular vesicles by electron microscopy. For negative staining, extracellular vesicles were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h. Then, 10 μL of samples were gently dropped on copper grids, and pre-coated with Formvar film (Ted Pella) for 2 min for deposition of the extracellular vesicles in the film. After that, the drop was gently dried with filter paper, incubated with 5% uranyl acetate for 30 s, and the copper grids were gently dried with filter paper. Finally, images were obtained in a Transmission Electron Microscope FEI Tecnai Spirit operating at 120 kV. In the second methodology, extracellular vesicles were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h. After fixation, extracellular vesicles were centrifuged at 20,000 × *g* for 10 min in an eppendorf centrifuge, washed twice in 0.1 M cacodylate buffer pH 7.2 and postfixed in a solution containing 1% osmium tetroxide, 1.25% potassium ferrocyanide, 5 mM calcium chloride in 0.1 M cacodylate buffer pH 7.2 for 30 min at room temperature in the dark. Then, extracellular vesicles were washed in the same buffer, dehydrated in increasing concentrations of acetone (30%, 50%, 70%, 90% and 100%), and embedded in Epon. Ultrathin