Colloids and Surfaces A xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Colloids and Surfaces A

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

Effect of the irradiation on Neuroblastoma-derived microvesicles: A physical and biological investigation

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ARTICLE INFO

Keywords:
Exosomes
Ionizing radiation
Neuroblastoma
SH-SY5Y
ζ-Potential
Radioresistence

ABSTRACT

Radiation therapy is one of the most commonly used therapeutic approaches for cancer treatment. Despite the undoubted benefits obtained with its application, numerous side effects occur, mainly related to the ability of irradiated cells to send messages to the un-irradiated ones (bystander effect). A key role in the transmission of bystander effects is played by micro-vesicles released from irradiated cells. Within this framework, particular attention was given to exosomes, vesicles with diameter smaller than 100 nm, whose composition consists in a cargo (protein and nucleic acids) and a double layer membrane (lipids and protein). Exosomes secreted by cancer cells after irradiation show different biochemical and biological features. The modification of secreted exosomes is suggested to be associated with unwanted radiotherapy effects. A major challenge is to establish the correlation between physical property and messages carried by micro-vesicles, for a potential use of exosomes as biomarkers for side effects of radiotherapy. In this work, we report that micro-vesicles secreted by X rays-irradiated neuroblastoma cells stimulate proliferation and confer radio-resistance, in a greater extent than those secreted by un-irradiated cells. The micro-vesicles were analyzed according to their morphology, size and their ζ-potential. The obtained results suggest that the surface charge distribution allows distinguishing the vesicles of the control neuroblastoma from that irradiated.

1. Introduction

Exosomes are small, secreted vesicles with a diameter of approximately 40–100 nm, released from cells both constitutively and following stimulation. They represent a sophisticated cellular double-layered product carrying complex signals such as proteins, mRNA, and miRNA [1]. Their unique "packaging" provides both protection of water-soluble cargo and membrane molecular machineries to sites even very far from their origin districts [2,3].

Although the discovery of these vesicles dates back to more than thirty years ago, their physiological function is still not clearly understood, albeit recent studies demonstrated that cancer cells-derived exosomal fraction has a different composition if compared to the fraction released from healthy cells of the same organism [4]. For this and other properties their use as biomarkers in cancer diagnosis is being intensively investigated to develop next-generation diagnosis technology for detecting silent human diseases with a low patient burden [5]. Moreover, a large number of in vitro studies demonstrated that cultured cancer cells induce their proliferation interacting each other via autocrine communication perpetuated by exosomes [6]. *In vivo* studies shown that tumor derived microvesicles play a crucial role in cancer progression acting via paracrine mechanisms to promote tumor-induced immune suppression, angiogenesis, and pre-metastatic niche formation [7,8].

The treatment of tumor with ionizing radiation is a commonly used strategy in the fight against cancer, although the side effects are very serious [9]. Cells exposed to ionizing radiation can deliver messages to un-irradiated cells, promoting an increase or even a decrease in recipient cells viability, depending to the cell type. This process, called bystander effect, can activate or depress defence mechanisms intervening in cell damage response [10,11].

The factors mediating communication between exposed and un-exposed cells can be transferred between cells via gap junctions or can be released into the extracellular media after irradiation [12,13]. In the last few years, microvesicles have been proposed to act as vehicles for intercellular information exchange in bystander effect [14,15]. Never-

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http://dx.doi.org/10.1016/j.colsurfa.2017.05.029
Received 28 February 2017; Received in revised form 9 May 2017; Accepted 12 May 2017

theless, the effect of IR on exosomes-mediated signalling has been studied in very few *in vitro* models and the results obtained are often controversial [16].

The presence of side effects resulting from radiotherapy is one of the major limitations of this therapeutic approach. The problem is accentuated by the lack of fast and reliable tests for assessing the individual's systemic response to radiation. In fact it is well known that the body's response to X rays varies with the genotype, age, sex and general state of the patient. The chemical-physical analysis of the circulating microvesicles after the patient's treatment can give a more complete picture of the individual radiation response. Evidently, understanding what is the mechanism by which radiation can induce undesired benefit to the tumor itself or otherwise, damage to healthy tissue, may open new perspectives to future therapeutic approaches. The aim of this work was to verify how the physical chemical nature of exosomes released by neuroblastoma cells is modified after irradiation of cells and if these changes interfere with the process of self-activation of tumour cells in culture.

2. Material and methods

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine and sterile materials are provided from Flow Laboratories (Irvine, UK); human neuroblastoma SH-SY5Y were gently provided by Katia Aquilano (Tor Vergata University, Rome). SH-SY5Y cells line was maintained in DMEM F-12 supplemented with 10% FBS, 50 U/mL penicillin/streptomycin and 2 mM L-glutamine in an atmosphere of 5% CO_2 and air at 37 °C. Unless otherwise specified, the reagents used are of Sigma (MI, USA)

2.1. Irradiation

Cells (70–80% confluence) were irradiated at a dose-rate of 1.1 Gy/min by using CHF 320 G generator (Gilardoni Mandello del Lario, LC, Italy) equipped with a Cu filter of 0.5 mm, operating at 250 KeV, 15 mA for increasing intervals of time in order to obtain increasing doses of X-ray (0,3–10 Gy).

2.2. Microvesicles purification

Microvesicles (EVs) were isolated by differential ultracentrifugation as previously described [17]. Each experimental point was consisting of EVs derived from 90 × 10^6 cells in 24 h. Medium was replaced by fresh media lacking serum two hours before cell irradiation. 24 h later conditioned medium (CM) was harvested and processed for EVs purification. Briefly CM was centrifuged at 300 x g for 10 min at 4 °C to pellet cell debris. Supernatant was centrifuged at 2000 × *g* for 20 min at 4 °C (2 K pellet), filtered with sterile filter unit (pore size 0,22 μm), transferred to new tubes, and finally centrifuged for 90 min at 100.000 x g (P100 pellet). All pellets were washed in 50–60 ml of PBS and recentrifuged at the same speed before being resuspended in 90 μl of sterile PBS. Cells pelletted from first centrifuge (300 x g) were pooled with cells detached by trypsin-EDTA treatment, and counted by Thoma cell counting chamber.

2.3. Western blot analysis

Cell pellets and isolated exosomes (P100 and P100*) were lysed in RIPA buffer (Sigma-Aldrich Co., MI, USA) containing 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and protease and phosphatase inhibitor cocktail (Roche, Mannheim Germany). Total protein concentration, in cell and exosome lysates, was determined by Lowry method [18]. Subsequently, samples were resuspended in loading buffer, boiled at 99 °C for 5 min and electrophoresed using SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Bio-Rad, Laboratories, USA), blocked with 5% non-fat dry milk in Tris buffered saline/Tween 20. Nitrocellulose membranes were stained with primary antibodies against monoclonal anti-human CD81 (clone B-11, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), monoclonal anti-human flotillin-1 (clone 18/Flotillin-1, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) polyclonal anti-human ALIX (Cell Signaling Technology, MA, USA), monoclonal anti-Calreticulin (Abcam, Cambridge, UK), monoclonal anti-GAPDH (Sigma-Adrich Co., MI, USA). All the primary antibody were used 1:1000 for Western blotting analysis. The appropriate peroxidase-conjugated secondary antibodies were incubated with membrane, using goat anti-rabbit-HRP, goat anti-mouse-HRP (Bio-Rad Laboratories, USA). All secondary antibodies were used at 1:5000 dilutions for Western blotting analysis. Immunoreactive bands and the relative intensity of protein bands were visualized by a Fluorchem Imaging System (Alpha Innotech, USA) upon staining with the ECL Western Blot Detection system from Amersham (Milano, Italy).

2.4. RNA isolation

RNA purification was performed as described by Chomczynski et al. [19], with some variations. Briefly, exosomes purified from conditioned medium of semiconfluent un-irradiated and irradiated cells (approximately 90 × 10^6 cells, as previously described), were homogenized in 1 ml of TRIzol reagent (Sigma-Aldrich Co., MI, USA). Subsequently, 200 μl of chloroform (Sigma-Adrich Co., MI, USA) per sample were added, samples have been shaked vigorously for 20 s, and incubated 5 min at room temperature. Samples were centrifugated at 12.000 x g for 20 min at 4 °C, colorless upper aqueous phase were collected and isopropyl alcohol were added (2:1 v/v) for precipitating RNA (Sigma-Aldrich Co., MI, USA). After 10 min of incubation at room temperature, samples were centrifugated at 12.000 x g for 15 min at 4 °C. Pellets containing RNA were washed with 1 ml of 75% ethanol and centrifugated at 7.500 x g for 5 min at 4 °C. Lastly, pellets were air-dried for few minutes and resuspended in 40 μl of RNAse free water. Spectrophotometric analysis was performed to determine samples concentration (absorbance 260 nm) and purity (absorbance 260/280).

2.5. Uptake of P100 by SH-SY5Y cells

P100 uptake was studied using immunofluorescence after labelling microvesicles with dye SYTO RNAselect green fluorescent stain (Molecular Probes, Eugeene Oregon USA). Briefly, after being resuspended in 100 μl of sterile PBS, exosomes have been labeled with the dye as described by the producer and after incubation, Exosome Spin Columns (MW 3000) (Life Technologies, Ltd) were used to remove the unincorporated dye from exosome labeling reactions. Cells, at 40–50% confluence, were plated on 12-mm glass cover-slips inserted in 16-mm wells and grown in complete DMEM F-12. One hour before incubation with labeled exosomes, medium was replaced by fresh media serum-free and cells coocultived with fluorescent vesicles for different times (30 min, 3 and 16 h) at 37 °C. Subsequently, cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde and, after permeabilization with Triton X-100 (0.1%), incubated with α-Tubulin (1:2000) (clone B-5-1-2, ascites fluid, Sigma-Aldrich Co., MI, USA) for one hour and then with donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody (1:500) (Life Technologies, Ltd), to visualize cell cytoskeleton. After one hour cells were incubated with DAPI (1/1000 in PBS) for nuclei staining. The slides were mounted with Fluormount (Diagnostic BioSystem, Pleasanton, CA.). The fluorescence observations were carried out using a confocal Laser Scanning Microscope (CLSM, Olympus FV1000 Corp., Tokyo, Japan), exciting at 488 nm with an Ar laser and 543 nm with a He laser. Slides were observed using a 25 x or 60 x (NA 1.35) objective and optical zooms varying from 1 x and 3x. Three-dimensional images were constructed from a series of 2-D cross-sectional images (x-y plane) captured at 0.5-μm intervals along the z-axis using IMARIS 6.2.0 software (Bitplane AG, Switzerland).