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Research paper

Transfer of multidrug resistance among acute myeloid leukemia cells via extracellular vesicles and their microRNA cargo

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ARTICLE INFO

Abbreviations:
EVs
extracellular vesicles
MRP-1
multidrug resistance protein 1
Pgp
P-glycoprotein 1
EVs/AR
extracellular vesicles generated by HL60/AR cells
EVs/S
extracellular vesicles generated by HL60 cells
HL60/EVs1
HL60 cells receiving one treatment with EVs generated by HL60/AR
HL60/EVs4
HL60 cells receiving four treatments with EVs generated by HL60/AR

Keywords:
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ABSTRACT

The treatment of acute leukemia is still challenging due in part to the development of resistance and relapse. This chemotherapeutics resistance is established by clonal selection of resistant variants of the cancer cells. Recently, a horizontal transfer of chemo-resistance among cancer cells via extracellular vesicles (EVs) has been suggested.

The aim of this research was to investigate the role of EVs in chemo-resistance in acute myeloid leukemia. For this purpose, the sensitive strain of the promyelocytic leukemia HL60 cell line was studied along with its multi-resistant strain, HL60/AR that overexpresses the multidrug resistance protein 1 (MRP-1). A chemo-resistance transfer between the two strains was established by treating HL60 cells with EVs generated by HL60/AR. This study reveals that EVs from HL60/AR can interact with HL60 cells and transfer at least partially, their chemo-resistance. EVs-treated cells begin to express MRP-1 probably due to a direct transfer of MRP-1 and nucleic acids transported by EVs. In this context, two microRNAs were highlighted for their high differential expression in EVs related to sensitive or chemo-resistant cells: miR-19b and miR-20a. Because circulating microRNAs are found in all biological fluids, these results bring out their potential clinical use as chemo-resistance biomarkers in acute myeloid leukemia.

1. Introduction

The treatment of acute leukemia is still challenging due in part to the development of resistance to initial chemotherapy and relapse [1]. The molecular mechanisms underlying chemotherapeutics resistance can involve activation of oncogenes or inactivation of tumor suppressor genes, changes in DNA damage response, changes in apoptosis pathway, specific changes in the treatment target and increase in drug export by cancer cells [2]. This last mechanism is mediated by drug efflux pumps located at cell membrane and expelling out of cells the xenobiotics, maintaining their intracellular concentration at a sub-lethal level. Among them, the multidrug resistance protein 1 (MRP-1) and/or the P-glycoprotein 1 (Pgp) can be expressed on cancer cells of acute myeloid leukemia (AML) patients and contribute to resistance to anthracyclines *in vitro* [3–5]. The clinical relevance of these experiments is still debated in the scientific world. Some studies correlated MRP-1 and Pgp overexpression with poor prognosis in AML [6,7], while others could not confirm this hypothesis [8].

Relapse or resistance to chemotherapy in acute myeloid leukemia is often due to a clonal selection of some resistant sub-populations of leukemia cells that would contribute to relapse after the treatment arrest [1]. In this process, the administered chemotherapy applies a

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selective pressure enabling the expansion of these chemo-resistant cells [9]. Recently, an alternative pathway involving a horizontal transfer of chemo-resistance among cancer cells via extracellular vesicles (EVs) has been suggested [10].

EVs are generated by almost all cell types and measure from 0.03 to 1 µm. "EVs" represents a generic term that designates heterogeneous vesicles known by different names depending on their tissue of origin, size or intracellular origin. In this way, these vesicles can be mentioned as microparticles, oncosomes, microvesicles, ectosomes, exosomes, and so on. Currently, there are still some difficulties to establish clear guidelines for EVs' denomination, partly due to the various generation and isolation methods used in the literature. Therefore, we chose here not to distinguish between EVs' types [11]. EVs are massively produced by cancer cells and contribute to the tumor microenvironment. EVs' cargo includes nucleic acids, membrane and cytosolic proteins, all originating from their producing cell. By interacting with cells and by transferring their content, these vesicles could modify target cells' phenotype [12]. In this way, EVs contribute to intercellular communication and play a role in tumorigenesis mechanisms such as metastasis, angiogenesis [13] and hypercoagulation [14]. EVs are found in all biological fluids such as saliva, urine [15], cerebrospinal fluid [16] and blood [17] and represent therefore good candidates in biomarkers research. As mentioned earlier, EVs could also transfer multidrug resistance from resistant cancer cells to sensitive ones. This mechanism would involve a direct transfer of the efflux pump responsible for the multidrug resistance. In this way, MRP-1 and Pgp have been shown to be expressed on EVs and transferred to recipient cells *in vitro* [18–20]. Nucleic acids such as messenger RNAs or microRNAs could also be transported by EVs and would impact target cells' phenotype, rendering them more resistant to chemotherapy [20,21]. This horizontal transfer has also been highlighted *in vivo* on a MCF7-murine xenograph model [22]. In this study, the breast cancer cells of the xenograph acquired Pgp expression after injection to the mouse of EVs from Pgp expressing cancer cells. This effect lasted at least two weeks.

The aim of this research was to investigate the role of EVs in chemo-resistance in AML. For this purpose, the sensitive strain of the promyelocytic leukemia HL60 cell line was studied along with its multi-resistant strain, HL60/AR that overexpresses MRP-1. The transfer of this resistance between the two strains was established by treating HL60 cells with EVs generated by HL60/AR (EVs/AR). The phenotype of the EVs-recipient cells was then analyzed. A particular interest was given to the microRNAs contained in EVs for their potential role in chemo-resistance transfer.

2. Materials and methods

2.1. Cell culture

The acute promyelocytic leukemia cell line (HL60) and its multidrug resistant strain (HL60/AR) were kindly provided by the URBC laboratory from the University of Namur, Belgium. These cells were cultured in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Biological Industries) and 1% penicillin/streptomycin 10,000 U (Lonza, Verviers, Belgium). Cells were re-cultured in exponential phase at a concentration of 250,000 cells per milliliter and maintained in a 5% CO_2 humidified atmosphere at 37 °C. HL60/AR cells were continuously treated with 100 nM daunorubicin (Cerubidine® Sanofi, Machelen, Belgium) in order to maintain their multidrug resistance.

2.2. Extracellular vesicles harvesting

To obtain EVs from conditioned medium, the cell suspensions were centrifuged 5 min at 200*g* and the resulting supernatant was further centrifuged 15 min at 2500 g to eliminate cells. EVs were then concentrated 6.7 times by a high-speed centrifugation. A 1ml-supernatant sample was placed in a microcentrifuge polyallomer tube (Beckman Coulter, Marseille, France) to undergo the high-speed centrifugation (100,000*g* 90 min at 4 °C) in an Optima MAX Ultracentrifuge with TLA-100.3 rotor (Beckman Coulter, Marseille, France). EVs isolated from the supernatant of HL60/AR and HL60 cells were designated EVs/AR and EVs/S respectively.

2.3. Treatment with extracellular vesicles for the functional tests

For the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cytotoxicity assays, the daunorubicin retention assays and the reactive oxygen species (ROS) generation assays, HL60 cells were treated with EVs/AR following this ratio: 1,000,000 cells treated with 200 µl of harvested EVs. HL60 cells receiving one treatment with EVs/AR for 20 h were mentioned as HL60/EVs1. These EVs/AR were isolated from a 72 h-conditioned medium. Another treatment course, in which cells were treated with EVs/AR four consecutive times (named HL60/EVs4), was also tested. Cells were incubated two following times with EVs/AR for 48 h and once for 72 h, followed by a 20 h-incubation. The EVs/AR used for these treatments were isolated from the conditioned medium of HL60/AR cells cultured in parallel.

2.4. Transmission electron microscopy (TEM)

Samples were prepared as previously demonstrated [23]. Briefly, HL60/AR cells and their 48-h produced EVs were pelleted by a high-speed centrifugation at 100,000*g* for 90 min, before being processed for TEM observations. The samples observation was performed using a Tecnai 10 TEM (FEI, Eindhoven, The Netherlands; resolution of about 5 nm at 80 kV).

2.5. EVs-tracking by PKH67

EVs/AR were harvested from 6 ml of a 72-h conditioned medium as explained earlier. The pelleted EVs were then labeled with the PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich, Saint-Louis, Missouri, USA) following the manufacturer instructions, with minor modifications. Briefly, EVs pellets were suspended into 500 µl Diluent C and 2 µl of PKH67 for a 5-min incubation at room temperature. 1 ml of bovine serum albumin (BSA) 1% was then added to stop the staining. EVs were finally rinsed with PBS (Lonza, Verviers, Belgium) by two sequential high-speed centrifugations. 1,000,000 HL60 cells were treated with stained EVs from 1.6 ml conditioned medium for 20 h and analyzed by flow cytometry. To take into account the potential residual EVs-unbound probe, a control was performed by the use of fresh medium instead of EVs for staining steps (PKH67 w/o EVs/AR). The results are represented as the percentage of fluorescence-positive cells arbitrary established at 5% of untreated cells. The BD FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA), with a 15 mW, 488 nm, argon-ion laser as light source and the FL1 emission filter, was used for flow cytometry measurements. BD CellQuest™ Pro Software (BD Biosciences, San Jose, CA, USA) was used for all data acquisition.

2.6. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cytotoxicity assay

The MTT assay was performed as previously described [24]. Briefly, a MTT solution (Sigma-Aldrich, Saint-Louis, Missouri, USA) was added to cell culture at a final concentration of 0.5 mg/ml. A 5-h incubation at 37 °C enabled the conversion of MTT into formazan crystal by alive cells. Finally, 150 µl of acidified isopropanol was added to dissolve the formazan crystals and the absorbance was measured using a plate reader (xMark™ Spectrophotometer – Bio-Rad) at 550 nm and 750 nm to correct for background noise. The results are expressed as a ratio between the optical density (OD) of the daunorubicin-treated well and the OD of the control well untreated with daunorubicin.