

Role of exosomes released by chronic myelogenous leukemia cells in the cross-talk with endothelial cells

INTRODUCTION

Chronic myeloid leukemia is a myeloproliferative disorder characterized by a reciprocal translocation t(9:22) (q34;q11) that leads to the formation of the Philadelphia (Ph) chromosome (1). This translocation leads to the expression of constitutive tyrosine kinase Bcr-Abl that, through downstream cascades, causes inhibition of apoptosis, increased proliferation, VEGF-mediated angiogenesis and altered adhesion of leukemic blasts to bone marrow (BM) microenvironment (1). This microenvironment is characterized by different populations of accessory stromal cells along with other factors including extracellular matrix and vessels.

In the literature there are a large number of evidences that associate BM also as a source of signals which modulate the growth, survival, tumor progression and resistance to therapies (2). An important field of investigation is represented by the study of BM microenvironment as active participant in resistance to therapies.

Microvesicles released by malignant cancer cells constitute a part of the tumor microenvironment, they can transfer several messages to target cells and may be critical to disease progression. Over the last few years, there has been an increase in the understanding of the cross-talk that occurs between many tumors and relative microenvironments at systemic, cellular and molecular level. As shown in literature not only soluble factors and chemokines have been implicated in the tumor formation, but tumor derived exosomes (TDEs) also play a crucial role in the regulation of tumor progression and in the metastatic mechanisms.

Exosomes are biological nanovesicles (40-100 nm) that are formed by the inward budding of multivesicular bodies (MVB), as a component of the endocytic pathway. They are released from different cell types under both normal and pathological conditions. They function as cell free messengers, playing a role in the cell-cell communication, and it was demonstrated that they transport proteins, mRNAs, lipids and miRNAs. These vesicles have pleiotropic functions in the regulation of tumor growth and progression, promoting immune escape, tumor invasion, angiogenesis and metastasis. In addition to effects exerted on the primary tumor microenvironment, TDEs also play a role in the establishment of the pre-metastatic niche.

It was recently showed that CML cell lines such as LAMA84 and K562 and Imatinib-resistant LAMA84 cells as well as patients leukemic blasts, release exosomes that directly affect endothelial cells thus modulating the process of neovascularization. In particular, the stimulation of HUVEC with CML exosomes activate signal transduction pathways leading to the release of IL 8 and the induction, *in vitro and in vivo*, of an angiogenic phenotype (1,3). A topic of particular interest is that exosomes contain miRNAs that can be shuttled to target cells and modulate their behavior.

MicroRNAs are a class of noncoding small RNAs of 21 to 25 nucleotides, generated from RNA precursors, that regulate expression of target mRNAs post-transcriptionally, binding to partially complementary 3' UTR of mRNA and causing target degradation or translation inhibition (4). MiRNAs are expressed in a tissue and cell-type specific manner and play essential roles in many biological processes, including proliferation, apoptosis and differentiation (5). In addition, aberrant miRNA expression is strongly implicated in cancer genesis and progression and accumulating evidences indicate that some miRNAs can function as oncogenes or tumor suppressors (6).

Interestingly, exosomes contain miRNAs and have been associated with cancers, including those of the lung, ovary and glioblastoma (7). Multivesicular bodies are functionally linked to miRNA effector complexes, perhaps indicating mechanisms for miRNA targeting to exosomes and the concept of exosome-mediated directed transfer of selected microRNA between cells is extremely attractive. In CML, Bcr-abl is known to upregulate the expression of oncogenic miRNAs; for example miR-17-92 cluster of oncomirs which facilitate tumour progression (8). Simultaneously, Bcr-abl downregulates the expression of tumour suppressor miRNAs like miR-203 (9). Recent works have demonstrated that CML cell lines (K562 and LAMA84) as well as leukemic blasts release exosomes and that the addition of the exosomes to endothelial cells (EC) stimulate an angiogenic phenotype through a src-dependent mechanism (1,10). It would be interesting to investigate the CML exosomes mediated cross-talk with endothelial cells to further characterizing the mechanisms by which CML-produced exosomes modulate the angiogenic process.

AIMS

The main objective of my research project is to further understand the role played by the CML produced exosomes in the establishment of a paracrine crosstalk between leukaemia cells and endothelial cells studying the expression of selected mRNA and the protein and miRNAs profiling of exosomes.

The experimental strategy will be based on the use of CML cell lines as LAMA84 or K562, on isolation of their exosomes and study of cross-talk with endothelial cell line as HUVEC.

The project will be articulated into different integrated steps:

- 1 Characterization of exosomes released by LAMA84/K562 cells through Western Blot and Biophysical assays;
- 2 Protein and miRNA profiling and study of LAMA84/K562 exosomes;
- 3 Study of cross-talk between exosomes released by LAMA84/K562 cells and endothelial cells;

AIM 1

Exosomes released by LAMA84/ K562 cells during a 24h culture period, will be isolated from conditioned culture medium supplemented with 10% FBS (previously ultracentrifuged) by different centrifugations. To further verify the identity of vesicles as exosomes, will be isolated exosomes on

a 30% sucrose/D2O cushion as described by Lamparski and colleagues. Vesicles contained in the cushion will be recovered, washed ultracentrifuging for 90 min in PBS and collected for use. The activity of acetylcholinesterase, an exosome marker protein, will be determined as described by Savina et al. Western blot analysis will be performed with exosome marker proteins such as CD63, ALIX or TSG101. The exosomes will be characterization also through Dynamic Light Scattering (DLS) in collaboration with Institute of Biophysics – CNR, Palermo.

AIM 2

The proteomic analysis will be carried out on LAMA84 and K562 cell lines. In order to obtain a proteome profile, will apply both shotgun and classic proteomic approaches, by using a high mass accuracy Orbitrap MS and two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) analysis. For the validation of the proteomic analysis, Western Blot assays for the selected proteins will be performed.

In order to test the levels of selected transcripts will be measured by reverse transcription (RT) and TaqMan real-time quantitative polymerase chain reaction (RT-PCR) and analyzed with Microsoft Excel software. The miRNA profiling of LAMA84/K562 cells and their exosomes will be carried out with Real Time PCR approach, miRNA cards or Nanostring Technology analysis.

Loss of function and gain of function

AIM 3

In order to test the *in vitro* effects of TDEs on endothelial cells, will be carried out proliferation, motility and angiogenesis assays.

Proliferation: In order to test the vitality of HUVECs treated with exosomes released by LAMA84/K562 cells will be performed MTT or BRDU assay. Methyl-thiazol-tetrazolium (MTT) assay will be done as briefly described: cells were plated in triplicate or quadruplicate at 1.5 per well and exposed to escalating doses of curcumin for up to different days. Means and standard deviations generated from 3 to 4 independent experiments are reported as the percentage of growth. Cell proliferation curves were derived from these data by using Microsoft Excel software.

Motility: Migration assays will be performed following standard protocols with Transwells. Briefly, HUVEC cells will be suspended in low serum medium in transwell chemotaxis above 8 μ m pore filters, and exposed to chemoattractants with increased amount of exosomes released by LAMA84/K562 cells. Filters will be removed after 6h, fixed in ethanol and stained with Diff-Quick (Medion Diagnostics GmbH, Düringen, Switzerland). Each cell line will be tested in three independent experiments; the number of migrating cells in five high-power fields per well will be counted at 400X magnification.

Angiogenesis: Matrigel will be used to test the effects of exosomes on *in vitro* vascular network formation. Exosomes will be added to HUVEC plated on Matrigel in endothelial basal medium containing 0.2% of FBS. Cells will be incubated for 6h and then evaluated by phase-contrast

microscopy and photographed. The length of the cables will be measured manually with the IMAGE-J software (<http://rsbweb.nih.gov/ij/>).

Conclusions

Any data obtained from this project could help to understand the biological and molecular events that are implicated in the cross-talk between CML-produced exosomes and endothelial cells in the angiogenic process. This study could help to increase the knowledge in CML progression and use it for the development and customization of treatments for patients with CML.

References

1. Role of exosomes released by chronic myelogenous leukemia cells in angiogenesis. Taverna S, Flugy A, Saieva L, Kohn EC, Santoro A, Meraviglia S, De Leo G, Alessandro R. *Int J Cancer*. 2012 May 1;130(9):2033-43.
2. Stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-expressing leukemia cells. Weisberg et al. *Mol. Cancer Ther* 2008; 7: 1121-9.
3. Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an Interleukin 8-dependent survival of leukemia cells. Corrado C, Raimondo S, Saieva L, Flugy AM, De Leo G, Alessandro R. *Cancer Lett*. 2014 Mar 18, S0304-3835(14)00154-2.
4. Herschkowitz JI and Fu X. MicroRNAs add an additional layer to the complexity of cell signalling. *Sci Signal* 2011; 4: jc5.
5. Sayed D and Abdellatif M. MicroRNAs in development and disease. *Physiol Rev* 2011; 91: 827-87.
6. Lovat F et al. MicroRNAs in the pathogenesis of cancer. *Semin Oncol* 2011; 38: 724-33.
7. Skog J et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; 10: 1470-6.
8. Venturini L et al. Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+cells. *Blood* 2007; 109: 4399-405.
9. Bueno MJ et al. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. *Cancer Cell* 2008; 13: 496-506.
10. Mineo M et al. Exosomes released by K562 chronic myeloid leukemia cells promote angiogenesis in a src-dependent fashion. *Angiogenesis* 2012; 15 :33-45.