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Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an Interleukin 8-dependent survival of leukemia cells

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ABSTRACT

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by the Bcr–Abl oncoprotein with constitutive tyrosine kinase activity. Exosomes are nanovesicles released by cancer cells that are involved in cell-to-cell communication thus potentially affecting cancer progression. It is well known that bone marrow stromal microenvironment contributes to disease progression through the establishment of a bi-directional crosstalk with cancer cells. Our hypothesis is that exosomes could have a functional role in this crosstalk. Interleukin-8 (IL 8) is a proinflammatory chemokine that activates multiple signalling pathways downstream of two receptors (CXCR1 and CXCR2). We demonstrated that exosomes released from CML cells stimulate bone marrow stromal cells to produce IL 8 that, in turn, is able to modulate both *in vitro* and *in vivo* the leukemia cell malignant phenotype.

1. Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder characterized by the Philadelphia (Ph) chromosome encoding the chimeric Bcr–Abl oncoprotein [1]. Bcr–Abl shows a constitutive tyrosine kinase activity that drives disease progression by stimulating a number of downstream signalling pathways [2]. Because of its critical role in CML pathogenesis, most therapies have focused on targeting Bcr–Abl [3]. However, while imatinib (IM), an ATP-competitive inhibitor of Bcr–Abl kinase, induces molecular remission in most patients, a number of investigation have demonstrated the persistence of a leukemic stem cell pool in the bone marrow (BM) niche even after IM treatment [4]. Furthermore, evidence have accumulated indicating that altered crosstalk between mesenchymal stromal cells (MSC) and CML cells may affect leukemia cell survival and resistance to chemotherapy [5,6]. Nevertheless, little is known about the possible mechanisms by which the BM microenvironment can modulate the progression of this disease.

Exosomes are nanovesicles released by cancer cells that can modulate the tumour microenvironment, promoting angiogenesis, tumour development and metastasis formation [7]. Peinado and colleagues have recently shown that cancer-derived exosomes modulate the crosstalk between malignant cells and the bone marrow microenvironment through the activation of the MET receptor tyrosine kinase [8]. Recent data from Kurre's group examined the role of exosomes released by acute myeloid leukemia (AML) cells in modulating cell signalling in the bone marrow microenvironment. They showed that both primary AML blasts and AML cell lines released exosomes enriched in microRNA relevant to AML pathogenesis [9]. We have recently showed that LAMA84 CML cells release exosomes and that the addition of those microvesicles to vascular endothelial cells (HUVEC) affects *in vitro* and *in vivo* angiogenesis though the stimulation of an Interleukin 8-mediated autocrine loop [10,11]. IL 8 belongs to the family of chemokines related to C-X-C motif and binds two seven transmembrane domain receptors, CXCR1 and CXCR-2 [12]. Interestingly, serum IL 8 levels have been found increased in hematologic malignancies compared to healthy controls [13] and increased expression of IL 8 and/or its receptors has been detected in cancer cells and stromal cells suggesting that IL 8 may modulate tumour microenvironment. However, the exact role of IL 8 in the growth of chronic myelogenous leukemia cells has not been investigated yet. Here we provide data showing that LAMA84-derived exosomes are able to activate bone

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marrow stromal cells which in turn release IL 8 acting as an *in vitro* and *in vivo* pro survival factor for chronic myelogenous leukemia cells.

2. Material and methods

2.1. Ethic statement

All animal experiments were conducted in full compliance with University of Palermo and Italian Legislation for Animal Care; Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi (DiBiMef) review board has approved this study.

2.2. Cell culture and reagents

Chronic myelogenous leukemia cell line, LAMA84, was obtained by DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 medium (Euroclone, UK). Bone marrow-derived stromal cell line, HS5, was obtained by ATCC (Manassas, VA, USA) and cultured in DMEM high glucose (Euroclone, UK). Both media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Euroclone, UK). Recombinant IL 8 (R&D Systems, MN, USA) was prepared at 10 ng/μl stock solution in sterile phosphate-buffered saline (PBS), aliquoted and stored at −80 °C. SB225002 (Cayman Chemical, Michigan, USA) was solubilized at 10 mM stock solution in DMSO and stored at −20 °C. Working dilutions were prepared in medium. All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), if not cited otherwise.

2.3. Exosome preparation

Exosomes released by LAMA84 CML cells after a 24-h culture period in presence of FBS previously ultracentrifuged (vesicle free media), were isolated from conditioned culture medium by differential centrifugation as described previously [10,11]. Exosome pellet was washed and then resuspended in PBS. Exosome protein content was determined by the Bradford assay (Pierce, Rockford, IL, USA).

2.4. Adhesion assay

Adhesion assays were performed as previously described [10]. Briefly, HS5 monolayer was pretreated in a 24-well plate, for 48 or 72 h with 50 μg/ml of LAMA84-derived exosomes or with exosome-deprived medium as negative control. After treatment, stromal monolayer was washed with PBS and 0.5×10^6 cells/well CML cells were left to adhere for 3.5 h at 37 °C. In different set of experiments, LAMA84 cells were stimulated with recombinant IL 8 (r-IL 8); in presence or not of different concentrations of SB225002 as indicated and in presence or not of conditioned medium of HS5 previously treated with LAMA84 exosomes. Adhered cells were stained with haematoxylin eosin, each test group was assayed in triplicate; 15 high power (400×) fields were counted for each condition.

2.5. Migration assay

Migration assays were performed in transwell chemotaxis chambers with 3 μm pore filters (NeuroProbe, Cabin John, MD, USA) [11]. Specifically, LAMA84 (0.5×10^6cells/ well) were resuspended in 500 μl of RPMI 1% FBS with or without SB225002 (600–1000–1200 nM), and exposed, as chemoattractant, to conditioned medium of HS5 previously treated with LAMA84-derived exosomes (50 μg/ml) for 6, 24, 48 or 72 h, as indicated. After 18 h at 37 °C, filters were removed, fixed in methanol and stained with Diff-Quick (Medion Diagnostics GmbH, Dudingen, Switzerland). Each test group was tested in three independent experiments; the number of migrating cells in five high-power fields per well were counted at 400× magnification.

2.6. RNA extraction and real time PCR

HS5 were grown in 12-well plates and treated with 50 μg/ml of LAMA84-exosomes for 6, 24, 48 or 72 h. RNA was extracted using the commercially available illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA). RT-QPCR was performed in 48-well plates using the Step-One Real-Time PCR system (Applied Biosystem, Foster City, CA, USA). Taqman gene expression assays for GAPDH (Hs99999905_m1) control and for IL 8 (Hs00174103_m1) were obtained from Applied Biosystems (Foster City, CA, USA). Real time PCR was performed in duplicates for each data point.

2.7. RT PCR

Conventional PCR was performed using cDNA from LAMA84 cells, obtained as described above. Primer sequences for CXCR1 were 5′TCCTGGGAAATGACACAGCA′3 and 5′AAGCCAAAGGTGTGAGGCAG′3 and for CXCR2 were 5′GGGCAACAATACAGCA AACT′3 and 5′GCACTTAGGCAGGAGGTCTT′3. The reaction conditions were 35 cycles of 95 °C 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension phase at 72 °C for 5 min. The PCR products were separated on a 3% agarose gel and stained with 5 μl ethidium bromide prior to examination under UV light.

2.8. ELISA

HS5 conditioned medium was collected from cells treated with 50 μg/ml of LAMA84-derived exosomes for 6, 24, 48 or 72 h. Conditioned medium aliquots were centrifuged to remove cellular debris and then IL 8 protein concentrations were quantified using the ELISA kit (R&D Systems, MN, USA), according to manufacturer's protocol.

2.9. Western blot

LAMA84 cells were treated or not for 2, 10 or 30 min with 10 ng/ml of r-IL 8 in presence or not of 1000 nM SB225002. Total protein cell lysate were obtained and analyzed by SDS–PAGE followed by Western blot as previously described [11]. Antibodies used in the experiments were: Akt, phospho Akt, ErK 1/2, phospho Erk 1/2, β-actin (all from Cell Signalling Technology, MA, USA); CXCR1/2 (Santa Cruz Biotechnology, CA, USA).

2.10. Colony formation assay

LAMA84 cells were plated in 6-well (2000 cell/ml/well) in Iscove's-methylcellulose medium (Methocult H4230, Stem Cell Technologies, Vancouver, Canada) containing or not r-IL 8 (2, 5, 10 ng/ml); in presence or not of SB225002 300–600 nM and in presence or not of conditioned medium of HS5 previously treated with LAMA84 exosomes. After 14 days of culture, LAMA84 colonies were observed by phase-contrast microscopy and photographed. The area of twenty LAMA84 colonies per condition were measured with the IMAGE-J software (http://rsbweb.nih.gov/ij/).

2.11. CML mouse xenograft

Male NOD/SCID mice four-to-five week old were purchased from Charles River (Charles River Laboratories International, Inc., MA, USA) and acclimated for a week prior to experimentation. Mice received filtered water and sterilized diet ad libitum. Animals were observed daily and clinical signs were noted. Mice were randomly assigned to four groups of seven. Each mouse was inoculated subcutaneously (sc) in the right flank with viable single cells (2×10^7) suspended in 0.2 ml of PBS with: (i) r-IL 8 (200 ng/mouse), (ii) SB225002 (1000 nM), (iii) PBS (vehicle) or (iv) r-IL 8 plus SB225002. The day of injection was considered as day 0. Treatments were repeated twice a week, in the tumour site. Tumour xenografts were measured and mice were weighed twice a week. Tumour volume was determined by calliper by using the following formula: $L \times W^2/2 = mm^3$ where L and W are the longest and shortest perpendicular measurements in millimeters, respectively. The same formula was used to calculate tumour weights assuming that 1 mm^3 = 1 mg. Animals were euthanized at day 50, tumour was prelevated and tumour weight was measured.

2.12. Statistics

Data were expressed as means ± SEMs of three independent experiments. Statistical analysis was performed by using a paired samples t test. Differences were considered to be significant when p values were smaller than 0.05.

3. Results

3.1. LAMA84-derived exosomes induce the expression of IL 8 in HS5

Recent data from our laboratory suggest that CML exosomes can modulate angiogenesis through the stimulation of an IL 8-mediated autocrine loop in endothelial cells. In order to evaluate if exosomes released from CML cells may affect the production of IL 8 in other cells composing the bone marrow microenvironment, we treated HS5 BM stromal cells with 50 μg/ml of exosomes and measured IL 8 expression. Fig. 1 shows that LAMA84 exosomes contain a little amount of IL 8, as previously demonstrated [10], but the treatment of HS5 with LAMA84-exosomes increases the expression of IL 8 mRNA (Fig. 1a) and its release in the HS5 conditioned medium (Fig. 1b) thus suggesting that IL 8 expression in bone marrow microenvironment is modulated by leukemia exosomes.