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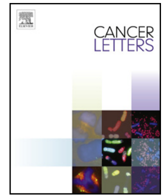

Original Articles

Induction of miR-146a by multiple myeloma cells in mesenchymal stromal cells stimulates their pro-tumoral activity

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Mutual communication between multiple myeloma (MM) cells and mesenchymal stromal cells (MSC) plays a pivotal role in supporting MM progression. In MM, MSC exhibit a different genomic profile and dysregulated cytokine secretion compared to normal MSC, however the mechanisms involved in these changes are not fully understood. Here, we examined the miRNA changes in human MSC after culture with conditioned medium of MM cells and found 19 dysregulated miRNAs, including upregulated miR-146a. Moreover, exosomes derived from MM cells contained miR-146a and could be transferred into MSC. After overexpressing miR-146a in MSC, secretion of several cytokines and chemokines including CXCL1, IL6, IL-8, IP-10, MCP-1, and CCL-5 was elevated, resulting in the enhancement of MM cell viability and migration. DAPT, an inhibitor of the endogenous Notch pathway, was able to abrogate the miR-146a-induced increase of cytokines in MSC, suggesting the involvement of the Notch pathway. Taken together, our results demonstrate a positive feedback loop between MM cells and MSC: MM cells promote the increase of miR146a in MSC which leads to more cytokine secretion, which in turn favors MM cell growth and migration.

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Introduction

In multiple myeloma (MM) disease, the growth and survival of cancer cells depend on factors secreted by the cellular compartment including stromal cells in the bone marrow (BM) [1]. Mesenchymal stromal cells (MSC) are self-renewable and multipotent progenitors which are involved in immunomodulation and homeostasis of the BM microenvironment [2]. Evidence has been provided that myeloma derived MSC (MM-MSC) show differences compared to MSC from healthy donors (ND-MSC), including a distinct genomic profile, dysregulated cytokine secretion (e.g. IL-6, VEGF, TNFα, IL1β) and impairment in osteogenic differentiation potential [3–6]. These features of MM-MSC favor MM growth and progression, whereas the mechanisms involved in the occurrence of these abnormal features have not been fully elucidated.

During MM development, MM cells attach to stromal cells in the BM and thereafter stimulate the latter to secrete more favorable factors which promote MM cell survival, proliferation, migration, and invasion, ultimately accelerating MM progression [1,7]. This interaction involves cell–cell contact and mutual stimulation through soluble factors, as well as exosomes which have been reported as a novel cell-to-cell communicator [8,9]. Exosomes are 40–100 nm diameter vesicles originating from late endosomes and released from numerous cell types [10]. They carry multiple cargo, including proteins, lipids, mRNA, and miRNAs, and mediate short- and long-range communication by delivering their functional molecules which induce various changes in recipient cells [10,11]. MM cells have been shown to secrete exosomes that can induce angiogenesis and osteoclast differentiation in the BM [12,13]. However, the effects of MM cell-derived exosomes on other BM-derived cells, including MSC, have not been studied.

MicroRNAs (miRNAs) are small, noncoding RNAs that negatively regulate gene expression by hybridizing to the sequences usually located at the 3′-untranslated region (UTR) of coding transcripts [14,15]. They are known to control numerous biological processes including inflammation, embryonic development, hematopoiesis,

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immune responses and tumorigenesis [14,16]. However, the role of a great number of these miRNAs is still unknown and contradictory results have been observed depending on the types of cells and tissues that are analyzed. Deregulation of miRNA expression is associated with gene expression profiling in MM and is involved in MM pathogenesis [17,18]. In addition, miRNAs play a critical role in self-renewal and differentiation of MSC [19,20]. As reported previously, MM-MSC exhibit a different miRNA profile than ND-MSC [21], but the functions of these deregulated miRNAs in MSC and MM progression are not fully understood.

In the present study, we examined the miRNA profile changes in MSC induced by MM cells and explored the possible role of exosomes in mediating miRNA delivery. Finally, we analyzed the functional effects of these altered MSC.

Material and methods

Cell culture

Bone marrow aspirates were obtained from the sternum of healthy donors after informed consent. The study was approved by the local ethical committee (NO. BUN14320097462). Bone marrow cells were separated by density gradient centrifugation with Ficoll-Hypaque (Nycomed, Lucron Bioproducts, De Pinte, Belgium), and the mononuclear cell fraction was collected. For primary MSC culturing, mononuclear cells were cultured in MesenPro medium (Invitrogen, Merelbeke, Belgium) containing 2% fetal calf serum (HyClone, Logan, UT, USA), 1% antibiotic/antimycotic (penicillin 10.000 U/ml; streptomycin 10 mg/ml, Lonza, Basel, Switzerland), 1% L-glutamine (Lonza) and 2% MesenPro growth supplement (Invitrogen). After 24 h, non-adherent cells were discarded, and adherent cells were continually cultured at 37 °C in 5% humidified CO_2. Human MSCs were used at passage 2–3 in this study. Human MM cell lines RPMI8226, OPM-2, LP-1, and U266 were cultured in RPMI 1640 medium (Lonza), supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, and antibiotics (Lonza).

miRNA array analysis

ND-MSC were cultured with conditioned medium obtained from RPMI8226 cells for 48 hours, and the cells were collected for total RNA isolation. Total RNA, including small RNAs, was extracted using the miReasy® Mini kit (Qiagen, Leusden, Netherlands). High-throughput miRNA expression profiling was performed by service provider Biogazelle (Ghent, Belgium). A validated miRNA screening pipeline was used for expression analysis of 755 microRNAs by means of real-time quantitative PCR with hydrolysis probe based miRNA assays.

RNA extraction and cDNA synthesis

Total RNA, including small RNAs and miRNAs, was extracted from MSC or MM cell-derived exosomes with the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription for miRNA was performed with the miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. cDNA for mRNA was synthesized from 500 ng total RNA using Thermo Scientific Verso™ cDNA synthesis kit (Thermo Scientific, Surrey, UK).

Quantitative real-time PCR (qRT-PCR)

miR-146a expression was quantified by real-time PCR and performed with the ABI 7900TH Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the reagents of the miScript SYBR Green PCR Kit (Qiagen) and specific primers of human miR-146a (MS00003535, Qiagen) and U6 (MS00033740, Qiagen). Relative miRNA expression normalized to U6 was carried out using the $2^{-\Delta\Delta Ct}$ method. Expression level of mRNA was quantified by qRT-PCR using ABI 7900TH Real-Time PCR System (Applied Biosystems). Specific primers for mRNAs can be found in Table 1. β-actin was included as an internal control. Relative mRNA expression normalized to β-actin was carried out using $2^{-\Delta\Delta Ct}$ method.

Exosome isolation

The human MM cells were cultured without serum for 24 hours and the conditioned medium was collected and filtered using a 0.22-μm pore filter. The filtered medium was concentrated using a Protein Concentrator (150 KD, Thermo Scientific) and filtered again with a 0.22-μm pore filter. Thereafter, exosomes were precipitated using ExoQuick-TC exosome precipitation solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instruction. Pelleted exosomes were used for total RNA and protein extraction.

Table 1
Primer sequences of genes evaluated by qRT-PCR.

Gene	Primer (forward) 5′-3′	Primer (reverse) 5′-3′
β-actin	ATGTGGCCGAGGACTTTGATT	AGTGGGGTGGCTTTTAGGATG
IL-6	GAGGATACCACTCCCAACAGACC	GCCCTCGCTTCCGTACTCG
CXCL1	AGCATCGCTTAGGAGAAGTC	GCAGGTAGAGTTAATCATACCCTTA
IP-10	CTACTGAGGTGCTATGTTCTTAGTG	GTACCCTTGGAAGATGGGAAAG
CCL5	GAAATGGGTTCGGGAGTACAT	AGGACAAGAGCAAGCAGAAA
Hes5	ACCAGCCCAACTCCAAGCT	GGCTTTGCTGTGCTTCAGGTA
Hey2	GAACAATTACTCGGGGCAAA	TCAAAAGCAGTTGGCACAAG

Abbreviations: IL-6: interleukin 6; CXCL1: chemokine (C-X-C motif) ligand 1; IP-10: interferon gamma-induced protein 10; CCL5: chemokine (C-C motif) ligand 5; Hes5: hairy and enhancer of split 5; Hey2: hairy/enhancer-of-split related with YRPW motif 2.

Transmission electron microscopy (TEM)

Transmission electron microscopy was performed as described previously [22]. Briefly, exosomes suspended in PBS were fixed in 2% paraformaldehyde and absorbed to a formvar-carbon coated grid. After adsorption, the samples were fixed again with 1% glutaraldehyde and negatively stained with 2% uranyl acetate. Grids were visualized on a TECNAI 10 transmission electron microscope (Philips, Amsterdam, The Netherlands) at 80 kV and the images were captured using iTEM software (Olympus Soft Imaging Solutions, Münster, Germany).

Uptake of exosomes

Exosome suspension was incubated with the cell membrane tracker DIO (Invitrogen) and re-pelleted using ExoQuick-TC exosome precipitation solution. The pellet was resuspended in PBS and unincorporated DIO in suspension was removed by Exosome Spin Columns (Life Technologies, Carlsbad, CA, USA). After incubation with DIO-labeled exosomes for 3 hours, MSC were stained with DAPI (Invitrogen) and images of exosome uptake were acquired with the Eclipse TE2000 fluorescence microscope (Nikon, Tokyo, Japan).

miR-146a mimics transfection

Human primary MSC were transfected with siRNA control or 50 nM miR-146a mimics (MSY0000449, Qiagen) using Lipofectamine™ RNAiMAX Reagent (Invitrogen). After 72 hours, the cells or supernatant were collected for further use.

Transfer of exosomes derived from MM cells transfected with Cy3-labeled miR-146a mimics

miR-146a mimics were labeled with Label IT siRNA Tracker Cy3 kit (Mirus, Madison, WI, USA) according to the manufacturer's instructions. MM cells were transfected with Cy3-miR-146a for 24 hours in medium with 5% exosomes-depletion serum (System Biosciences) and the transfection efficiency was analyzed using a FACSCanto flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the Eclipse 90i fluorescent microscope (Nikon). MM cells without microRNA transfection were included as a mock. After 48 hours, the exosomes were isolated from the mock and transfected cells, and incubated with ND-MSC for 3 hours. DIO and DAPI were added for MSC membrane and nucleus staining respectively. The cells were observed under the laser scanning microscope LSM 710 (Carl Zeiss, Oberkochen, Germany) and the pictures were captured using ZEN software (Carl Zeiss).

Enzyme-linked immunosorbent assay (ELISA)

IL-6 ELISA was performed according to manufacturer's instructions (eBioscience, San Diego, CA, USA). The absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Cell viability assay

MSC seeded in a 96-well plate were transfected with siRNA control or miR-146a mimics for 72 hours, and the cell viability was examined by a Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Luminescence was measured using a Glomax luminometer (Promega). MM cells were cultured in a 96-well plate with conditioned medium from MSC transfected with siRNA control or miR-146a mimics for 24 hours and the cell viability was determined by Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

Human cytokine array

Supernatant obtained from MSC transfected with siRNA control or miR-146a mimics were tested for cytokine levels using the Human Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly,

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