Leukemia Research 57 (2017) 20–26

Contents lists available at ScienceDirect

Leukemia Research

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

Bone marrow mesenchymal stem cells regulate stemness of multiple myeloma cell lines via BTK signaling pathway

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ARTICLE INFO

Article history:
Received 12 September 2016
Received in revised form 18 February 2017
Accepted 22 February 2017
Available online 24 February 2017

Keywords:
Bone marrow mesenchymal stem cells
Stemness
Bruton tyrosine kinase
PCI-32765
Multiple myeloma

ABSTRACT

Bone marrow mesenchymal stem cells (BM-MSCs) are key components of bone marrow microenvironment. Although the importances of BM-MSCs activation in myeloma cells growth, development, progression, angiogenesis are well known, their role in the regulation of myeloma stemness is unclear. In this study, myeloma cell lines (LP-1, U266) were co-cultured with BM-MSCs, we found that BM-MSCs could up-regulate the expression of key stemness genes and proteins (OCT4, SOX2, NANOG) and increase clonogenicity. Similarly, the mechanisms underlying the BM-MSC activation of myeloma stemness remain unclear. Here, we found that PCI-32765, a Bruton tyrosine kinase (BTK) inhibitor, treatment significantly down- regulate expression of key stemness genes and proteins in vitro co-culture system. Together, our results revealed that BM-MSCs could increase myeloma stemness via activation of the BTK signal pathway.

1. Introduction

Multiple myeloma (MM) is a hematological malignancy, mainly including osteolytic lesions, aneima, renal dysfunction, hypercalcemia, infection and other symptoms. In recent years, with the bortezomib, thalidomide, lenalidomide targeted drug application, the overall survival (OS) and progression-free survival (PFS) of patients have significantly improved [1–3]. However, MM remains incurable. This is associated with multiple myeloma stem cells (MMSCs). MMSCs are a small, self-renewing population, which are the roots of cancer and sources of therapy resistance [4,5].

MSCs are a kind of multipotent adult stem cells that present the stem cells ability such as extensive self-renewal, indefinite proliferation and multi-lineage differentiation [6]. Increasing studies have shown that BM-MSCs could stimulate myeloma cells growth, invasion, angiogenesis and drug resistance [7–10]. However, whether BM-MSCs increase myeloma cells stemness or not and the mechanism has not been reported yet.

Bruton tyrosine kinase (BTK), a member of the TEC tyrosine protein kinase family, plays a critical role in the survival and proliferation of B cells through activation of the B-cell receptor (BCR) signaling pathway on binding to special receptors in the downstream (such as growth factors, B cell antigen, chemokines, et al.). Activated BTK phosphorylates PLC-γ2 through its SH5 domain binding protein BLNK/SLP65, it further leading to activation of MAPK, p38, MEK/ERK, NK/SAPK and AKT signaling pathways [11]. As is well known, it was related to the development of B-cell malignancies including acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), follicular lymphoma (FL), mantle cell lymphoma (MCL) and diffuse large B cell lymphoma (DLBCL) [12–17]. Recent studies showed that there exists expression of BTK in myeloma cells and have been reported to predict poor prognosis [18]. In addition, PCI-32765, a small molecule BTK inhibitor, has been reported that inhibited myeloma cells survival, growth and migration [19]. Another second-generation BTK inhibitor, CGI1746, decreased expression of stemness genes and reduced tolerance to myeloma drugs [20]. Collectively, these findings suggested that BTK signaling pathway played important roles in the survival, growth and maintenance of stemness.

Abbreviations: BM-MSCs, Bone marrow mesenchymal stem cells; MM, multiple myeloma; BTK, Bruton tyrosine kinase; MMSCs, multiple myeloma stem cells; OS, overall survival; PFS, progression-free survival.

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http://dx.doi.org/10.1016/j.leukres.2017.02.005
0145-2126/

Thus, the purpose of our study was to investigate whether BM-MSCs influence myeloma cells stemness and to determine whether the BTK signalling pathway involves in this process.

2. Materials and methods

2.1. Myeloma cell lines

The human MM cell lines LP-1 and U266 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). LP-1 was cultured in RPMI1640 medium (Gibco), which contained 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 ug/mL streptomycin (Gibco). U266 was cultured in RPMI1640 medium (Gibco), which contained 15% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 ug/mL streptomycin (Gibco). All cells were cultured in a humidified atmosphere of 5% CO2 at 37 °C.

2.2. Isolation, culture of bone marrow mesenchymal stem cells

Marrow blood was collected from healthy donors and MM patients. Informed consent was obtained from all donors. Bone marrow mononuclear cells were obtained using Ficoll (Tian Jin Hao Yang biological manufacture Co. Ltd) and then were cultured in DMEM/F-12 medium (Gibco), which contained 15% fetal bovine (Gibco), 100 U/mL penicillin, and 100 ug/mL streptomycin (Gibco). The half medium was changed firstly after 48–72 h. Later, medium was changed every 2 or 3 days. The adherent cells were preserved and cultured in a humidified atmosphere of 5% CO2 at 37 °C.

2.3. Co-culture experiments

For co-culture experiments, (1) 5×10^5 LP-1 or U266 cells directly contacted with 3×10^5 BM-MSCs in six-well plates (2) 5×10^5 LP-1 or U266 cells were seeded in the upper chamber of transwell, while 3×10^5 BM-MSCs in the lower chamber. After 72 h, LP-1 and U266 cells were collected for further experiments.

2.4. Antibodies and chemicals

β-actin (BE5), OCT-4A (C30A3), SOX2(D6D9), NANOG(D73G4) antibodies were purchased from Cell Signaling Technology Company. BTK inhibitor PCI-32765 was purchased from Sigma Company.

2.5. Quantitative RT-PCR

Total RNA was extracted from LP-1 cells and U266 cells using the TRIzol reagent (Invitrogen). Then, 1 μg total RNA was reverse transcribed into cDNA using 5 × All-In-One RT MasterMix (Cat#G490 Abm) following the manufacturers' protocol. RT- PCR was carried out using a CFX96 RT-PCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR Master Mix (DBI® Bioscience) according to the manufacturer's instructions. Primer sequences for the GAPDH, OCT4, SOX-2, NANOG and BTK were provided by Beijing Aoke peak Biotechnology Co. Ltd. (Table 1)

Table 1
Primer sequences used in experiment.

Gene	Sequence
GAPDH	5′-TTCGTCATGGCTGTGAACCA-3′
OCT4	5′-AGCCCTCATTTCACCAGGCC-3′
SOX-2	5′-ACAACTCGGAGATCAGCA-3′
NANOG	5′-ACTGTCTCTCCTCTTCCTTC-3′
BTK	5′-GGCCGAAATCAGATACTTTAAC-3′

2.6. Western blot analysis

Cells were washed two times with ice cold PBS and lysed in Radio Immunoprecipitation Assay (RIPA) buffer (Solarbio) and 1 mM PMSF (Solarbio) on ice. Protein supernatants were collected and were measured using Bicinchoninic Acid (BCA) Protein Assay Kit (Solarbio). The equal quantity protein was separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Then, 5% the bovine serum albumin (BSA) was used to block of the rest points which are not linked. Subsequently, the membranes were incubated with appropriately diluted primary antibodies β-actin (BE5), OCT-4A (C30A3), SOX2 (D6D9) and NANOG (D73G4) to overnight at 4 °C. After that, the membranes were incubated with the secondary antibody rabbit anti-mouse IgG (Zhongshan Company, Beijing) for 1 h at room temperature. In the end, Image J software was used to analyze images.

2.7. Soft agar clonogenicity assay

Soft agar clonogenicity assay was carried out in a six-well plate and each well was covered with two layers of agarose, low gelling temperature (A9045, SIGMA-ALORICH, USA). 5×10^3 LP-1 or U266 cells were seeded in the upper layer, which contained 0.4 ml 0.7% agarose and 0.4 ml RPMI 1640 (20% or 30% fetal bovine serum, 200 U/mL penicillin, and 200ug/mL streptomycin). The lower layer contained 0.4 ml 1.2% agarose and 0.4 ml RPMI 1640 (20% or 30% fetal bovine serum, 200 U/mL penicillin, and 200 ug/mL streptomycin). After 21 days, colonies consisting of more than 50 cells were counted under inverted microscope.

2.8. Statistical analyses

Student's *t*-test was used for group comparisons. The p value ≤ 0.05 were considered statistically significant.

3. Results

3.1. Morphology of bone marrow mesenchymal stem cells

Results showed that we could get more adherent cells after 10 days and these cells showed typical morphological features of fibroblast cells (Fig. 1). Early studies found these fibroblast cells were exhibited clonal proliferation and the concept of MSC was proposed [21,22]. Unfortunately, there is still lack of a defined surface markers to identify MSCs in situ. In 2005, the Committee of Mesenchymal Stem Cells and Tissues of the International Soci-

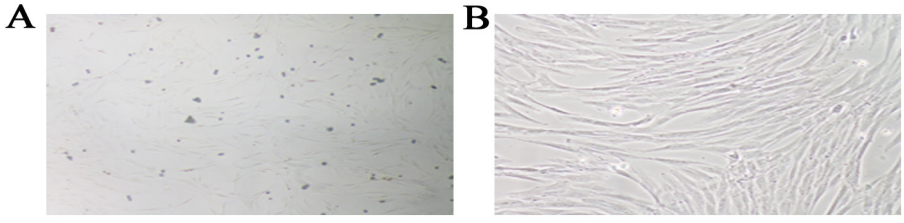

Fig. 1. Morphology of bone marrow mesenchymal stem cells under inverted microscope A. (magnification 40×) B. (magnification 100×).