RANKL expression in myeloma cells is regulated by a network involving RANKL promoter methylation, DNMT1, microRNA and TNFα in the microenvironment

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We studied the regulation of RANKL expression in myeloma by promoter DNA methylation. Methylation-specific polymerase chain reaction showed complete methylation of RANKL promoter in WL-2 myeloma cells but partial methylation in eight other lines. 5-AzaC treatment of WL-2 cells led to demethylation and re-expression of RANKL. Transwell and contact co-culture of WL-2 cells with normal bone marrow-derived mesenchymal stromal cells (BMSCs) resulted in comparable repression of DNA methyltransferase-1 (DNMT1) and re-expression of RANKL in WL-2 cells. Moreover, treatment of WL-2 cells with TNFα led to repression of DNMT1 and re-expression of RANKL in association with upregulation of miR-140-3p and miR-126, which are partially offset by addition of anti-TNFα antibody to transwell-coculture of WL2 with BMSC. Taken together, our results showed that TNFα in the marrow microenvironment led to RANKL demethylation and re-expression in myeloma cells through DNMT1 repression and upregulation of miR-126-3p and miR-140, both known to repress DNMT1 translation.

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1. Introduction

Multiple myeloma (MM) is characterized by clonal proliferation of malignant plasma cells in the bone marrow, associated with osteolytic bone lesions. Previous studies showed that the receptor for activation of the nuclear factor-κB ligand (RANKL) is essential for inducing osteoclast differentiation and activity [1]. Moreover, clinical studies have shown that RANKL level was significantly increased in the serum and bone marrow (BM) plasma of MM patients [2, 3]. Early studies suggested that RANKL is principally expressed by bone marrow mesenchymal stromal cells (BMSCs) and osteoblasts. However, recent studies indicated that RANKL could be secreted by myeloma cells, and RANKL secreted by myeloma cells could induce osteoclast formation, similar to RANKL derived from BMSCs or osteoblasts [4, 5]. Furthermore, RANKL expression level in myeloma cells is significantly correlated with the extent of osteolytic myeloma bone disease [3, 6, 7]. Therefore, RANKL in the BM microenvironment plays a pivotal role in osteolytic myeloma bone disease.

In MM, the BM microenvironment plays an essential role in myeloma plasma cell proliferation, migration, survival and drug resistance [8]. Moreover, it has been suggested that the BM microenvironment stimulated RANKL expression from BMSCs when co-cultured with myeloma cells [9, 10]. However, whether the microenvironment is involved in the expression of RANKL in myeloma cells remains unclear.

Promoter DNA methylation is characterized by the addition of a methyl group to the carbon 5 position of cytosine ring in promoter-associated CpG islands, which is associated with gene silencing [11]. However, DNA methylation is dynamic and reversible. Moreover, recent studies indicated that DNA methylation is involved in the regulation of RANKL expression [12]. Herein, we study whether the microenvironment may influence the RANKL expression in MM cells, and thereafter the mechanisms involved.

2. Material and methods

2.1. Cell culture

2.1.1. Human BMSCs

Bone marrow mononuclear cells from healthy bone marrow transplantation donors were obtained with written informed consent under approval of Institutional Review Board (The Hong Kong University and Hong Kong West Cluster Hospitals). Normal BMSCs were
harvested using standard Ficoll-Hypaque density sedimentation methods then expanded and characterized in vitro according to our previous published method [10].

2.1.2. MM cell lines

Culture conditions of WL-2 and 8 other myeloma cell lines are described in the supplemental material.

2.1.3. Co-culture of MM and BMSCs

A serial co-culture experiment was used, 2 × 10⁶/well WL-2 cells were placed in a transwell insert (0.4 μm pore size; Coring Costar, Cambridge, MA) to confluent BMSCs (2 × 10⁵/well), or added directly to BMSCs. To separate the MM and BMSCs in contact co-culture, co-cultures were harvested from myeloma cells using a positive immunoselection with anti-CD138 mAb (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) at the end of culture period [13,14].

Fig. 1. Promoter methylation and expression of RANKL. (A) M-/-U-MSP shows that the WL-2 myeloma cells (lane 9) and methylated control [PC] (lane 10) are totally methylated, while JNJ-3 (lane 1), KMS-12-PE (lane 2), MMX-8 (lane 3), NCI-H929 (lane 4), OCL-MYS (lane 5), OPM-2 (lane 6), RPMI-8226 (lane 7), U266 (lane 8) are partially methylated. Lane 11 is negative (no template) control. (B) M-/-U-MSP analysis of RANKL promoter methylation status in WL-2 cells (lane 1), and 1.5 μM 5-AzadC treatment resulted in demethylation of RANKL promoter in WL-2 cells (lane 2). Methylated control (lane 3) and negative control (lane 4). (C) qRT-PCR analysis of the RANKL expression in WL-2 cells. 5-AzadC treatment led to re-expression of RANKL expression in WL-2 cells. (D) Transwell co-culture of WL-2 cells and BMSCs resulted in demethylation of RANKL promoter in WL-2 cells. M: marker; lane 1: WL-2 cells; lane 2: transwell co-culture of WL-2 cells and BMSCs; lane 3: positive control; lane 4: negative control (reagent blank). (E) RANKL expression in WL-2 cells after transwell or contact co-culture with BMSCs.
To determine the influence of TNFα on WL-2 cells, different concentrations of recombinant human TNFα (Sigma, St. Louis, Mo.) were added to the culture medium of WL-2 cells.

To verify the effect of TNFα, WL-2 cells were pretreated for 20 min in the presence of anti-TNFα monoclonal antibody (1 μg/ml, R&D Systems, Minneapolis, MN) or anti-immunoglobulin G (IgG) control, and these antibodies were maintained throughout the transwell co-culture period.

3. Methylation-specific polymerase chain reaction and quantitative bisulftite pyrosequencing

DNA was extracted from JJN-3, KMS-12-PE, MOLP-8, NCI-H929, OCL-MY5, OPM-2, RPMI-8226, U266 and WL-2 myeloma cell lines by standard method. Bisulftite conversion was performed using EpiTect Bisulfite Kit (Qiagen). Primers and condition of MSP and pyrosequencing are detailed in supplementary material.

Fig. 2. Mechanism study of RANKL promoter demethylation in WL-2 after co-culture with BMSCs. (A) Expression of molecules implicated in DNA demethylation including DNMT1, AID and GADD45a in WL-2 cells (lane 1), WL-2 cells transwell co-cultured with BMSCs (lane 2) and WL-2 cells in contact co-culture with BMSCs (lane 3). (B) DNMT1 expression was determined using Western blot after treatment of WL-2 cells with varying concentrations of recombinant TNFα protein for 6 days. (C) RANKL expression in WL-2 cells after treatment with recombinant TNFα protein. (D) miR-126 and miR-140-3p expression in WL-2 cells after treatment with recombinant TNFα protein using stem-loop qRT-PCR analysis. (E) RANKL expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG. (F) miR-126 and miR-140-3p expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG. (G) DNMT1 expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG, representative Western blot result is shown.
3.1. 5-Aza-2’-deoxyuridine (5-AzadC) treatment

WL-2 cells were seeded in six-well plates at a density of $1 \times 10^6$ cells/ml and cultured with 1.5 μM of 5-AzadC (Sigma-Aldrich, St. Louis, MO) for 6 days.

3.1.1. Quantitative PCR

RNA was isolated, reverse transcribed for quantitative real-time PCR (qRT-PCR) of RANKL, mir126 and mir140-3p. GAPDH and SNORD48 were used as reference for RANKL and microRNA using the $2^{-\Delta\Delta Ct}$ method.

3.1.2. Western blot

Western blot was carried out as previously described using anti-DNMT1, -GADD45a, -AID and β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) [15].

4. Results and discussion

A previous study showed that methylation of the CpG island located between −260 bp to +615 of the TSS correlated with RANKL expression [12], and hence studied herein. MSP showed complete methylation of RANKL in WL-2 cells and positive control, and partial methylation in JIN-3, KMS-12-PE, MOLP-8, NCI-H929, OCL-MYS, OPM-2, RPMI-8226 and U266. (Fig. 1A, MSP primer target on −48 to 107 bp). Furthermore, complete methylation of RANKL promoter in WL-2 cells was associated with undetectable RANKL expression determined by qRT-PCR (Fig. 1C). Incubation of WL-2 cells with 1.5 μM of 5-AzaC resulted in demethylation of RANKL promoter as evidenced by emergence of the U-MSP signal (Fig. 1B), which was associated with re-expression of RANKL as evidenced by qRT-PCR (Fig. 1C). Therefore, WL-2 cells were utilized to explore the mechanisms of promoter DNA methylation regulating expression of RANKL in myeloma.

Using a transwell co-culture system with WL-2 cells and BMSCs, we investigated whether the microenvironment affected RANKL promoter methylation and expression in WL-2 cells. After 6 days of transwell co-culture, partial demethylation of RANKL promoter occurred (Fig. 1D), accompanied by upregulation of RANKL expression (Fig. 1E). To see if contact co-culture might lead to additional upregulation of RANKL, WL-2 cells were cultured with BMSCs in a contact co-culture manner, which yielded RANKL expression comparable to that of transwell co-culture (Fig. 1E). These results suggested that soluble factors in the microenvironment were primarily involved in RANKL promoter demethylation and expression in WL-2 cells, which was not enhanced by cell adhesion through contact co-culture.

DNA demethylation may reverse epigenetic silencing by promoter DNA methylation. Recent studies revealed that demethylation of methylated CpG dinucleotides can be achieved by a passive or active process [16]. Passive DNA demethylation is mediated by inhibition of DNMT1, and hence unmethylated alleles are generated during cellular replication. On the other hand, active DNA demethylation of methylated CpG dinucleotides is catalyzed by several enzymes [17] including GADD45a, activation-induced deaminase (AID), ten–eleven translocation (TET) and elongator complex protein 3 (ELP3). To explore the mechanism of demethylation of the RANKL promoter in WL-2 cells, we determined the passive and active demethylation related protein expression after the transwell and contact co-culture of WL-2 cells with BMSCs. Our results showed that levels of GADD45a and AID remained unaltered after transwell or contact co-culture (Fig. 2A). By contrast, DNMT1 expression was significantly reduced in WL-2 cells after the transwell co-culture with BMSCs for 6 days. However, contact co-culture of WL-2 cells and BMSCs did not yield additional DNMT1 downregulation compared with transwell co-culture (Fig. 2A). The expression of TET-1 isn’t detected in WL-2 cells and BMSCs (data not shown), and since ELP3 has been shown to lead to active demethylation of zygotic paternal genome rather than gene-specific demethylation [18], ELP3 was not examined in the present experiment.

The effect of transwell co-culture of WL-2 cells and BMSCs resulting in downregulation of DNMT1 in WL-2 cells suggested a paracrine cytokine mechanism. Of the cytokines secreted by myeloma BMSCs, a literature review showed that TNFα has been reported to be involved in DNA demethylation [19]. To verify the role of TNFα in RANKL demethylation and re-expression in myeloma cells, WL-2 myeloma cells were treated with recombinant TNFα. A previous study showed that the median concentration of TNFα in the BM plasma of myeloma patients was about 0.35 ng/ml, and the supernatant of myeloma cells co-cultured with BMSCs ranged from 0.118 to 0.152 ng/ml [20]. Therefore, WL-2 cells were cultured with recombinant TNFα at concentrations between 0.1 and 1.0 ng/ml, which led to downregulation of DNMT1 expression (Fig. 2B) and upregulation of RANKL expression (Fig. 2C) in WL-2 cells after 6 day treatment.

On the other hand, mir-140-3p and mir-126 [21,22], which are regulated by TNFα, have been shown by luciferase assays to directly target and repress DNMT1 translation [23,24]. Consistent with these data, we showed that mir-140-3p and mir-126 expression was upregulated by treatment of WL-2 cells with recombinant TNFα (Fig. 2D). As previous studies have shown secretion of TNFα in the supernatant of transwell-coculture of myeloma cells with BMSCs [19], anti-TNFα antibody was added to the transwell co-culture of WL-2 with BMSCs, which partially abrogated the co-culture-induced RANKL expression (Fig. 2E) and upregulation of miR-140-3p and miR-126 (Fig. 2F), and offset DNMT1 downregulation (Fig. 2G) in WL-2 cells. Furthermore, our pyrosequencing results showed the anti-TNFα antibody could attenuate the co-culture induced demethylation in WL-2 cells (Fig. S1). Taken together, the above results testified to the role of TNFα in the induction of demethylation and re-expression of RANKL in myeloma cells via upregulation of miR-126 and -140-3p, which may lead to translational inhibition, and hence downregulation, of DNMT1.

In conclusion, in the BM microenvironment, RANKL expression in myeloma cells is mediated by RANKL promoter demethylation induced by downregulation of DNMT1, which is associated with upregulation of mir-126 and -140-3p. TNFα plays a pivotal role in this paracrine mechanism.

Authorship contributions

CSC and LQY designed the study and analyzed the data. LQY conducted the experiments and helped in sample collection and clinical data retrieval. GCC and KLF helped in data analyses. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.05.010.

References


