Adiponectin inhibits the differentiation and maturation of osteoclasts via the mTOR pathway in multiple myeloma

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Abstract. The present study sought to investigate the correlation between adipose cytokines (visfatin, leptin and adiponectin) and markers of multiple myeloma bone disease, and to determine the effects and mechanism of action of adiponectin on the differentiation and maturation of osteoclasts in multiple myeloma (MM). The levels of visfatin, leptin and adiponectin were measured. Their association with the indices of myeloma tumor load and bone disease were analyzed. Reverse transcription-quantitative PCR was used to detect the expression of receptor activator of nuclear factor-κB ligand (RANKL), osteoclast associated Ig-like receptor (OSCAR), tartrate-resistant acid phosphatase (TRAP) and Cathepsin K genes. Flow cytometry was used to detect the expression of adiponectin receptor 1 (AdipoR1) and the phosphorylation of the mechanistic target of rapamycin kinase (mTOR) pathway-associated proteins mTOR and eukaryotic translation initiation factor 4E-binding protein (4EBP1). There were no significant correlations among leptin, visfatin and the indexes of myeloma tumor load and bone disease. Serum adiponectin levels were significantly lower in patients with newly diagnosed multiple myeloma compared with healthy volunteers (12.37±3.13 vs. 13.80±0.95; P<0.05). The number of mature osteoclasts in the adiponectin group was lower compared with in the control group. Adiponectin also inhibited the mRNA expression of the osteoclast-associated factors RANKL, OSCAR, TRAP and Cathepsin K. Comparison between the non-adiponectin group and the adiponectin group revealed that adiponectin increased the expression of AdipoR1 on the surface of osteoclast precursor cells (26.21±4.27% vs. 29.86±6.23%; P<0.05) and reduced the expression of phosphorylated (p-)mTOR (7.89±1.00% vs. 5.91±1.26%; P<0.05) and p-4EBP1 (26.78±5.00% vs. 22.49±4.24%; P<0.05). The p-mTOR and p-4EBP1 levels in the adiponectin + MHY1485 (an mTOR signaling pathway-specific agonist) group were significantly higher compared with those in the adiponectin group. It was revealed that adiponectin may inhibit osteoclast differentiation and maturation via the mTOR pathway. In conclusion, adiponectin inhibits the differentiation and maturation of osteoclasts by increasing the expression of AdipoR1 and reducing the phosphorylation levels of mTOR and 4EBP1 in patients with MM.

Introduction

Multiple myeloma (MM) is a hematological malignancy of terminally differentiated plasma cells. Myeloma bone disease (MBD) is the most common complication in patients with MM (1). Adipose cytokines including visfatin, leptin and adiponectin have been implicated in the stimulation or inhibition of tumor cell growth (2-5). For example, in a prospective study of 174 patients with MM and 348 controls (6), adiponectin was revealed to be associated with an increased MM risk. In contrast, Medina et al (7) demonstrated that adiponectin had an anti-proliferative effect on MM cells that was mediated by the protein kinase A/adenosine monophosphate-activated protein kinase (AMPK) signaling pathway. Adiponectin also was revealed to prevent MBD in a mouse myeloma model (8).

Little is known about the impact of adiponectin on bone disease induced by MM. To study this question, the present study aimed to determine the concentrations of visfatin, leptin and adiponectin in the serum and bone marrow and elucidate whether correlations exist between these concentrations and bone disease in patients with MM.

Osteoclasts are large multinucleated cells (9,10) that are derived from tartrate-resistant acid phosphatase (TRAP)-positive monocyte-osteoclast precursor cells [mostly cluster of differentiation 14 (CD14)+ mononuclear cells (11)] through the action of receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Osteoclast activation is associated with the development of MBD (12). For this reason, the present study...
investigated the effects of adiponectin on the differentiation and maturation of osteoclasts in MM.

Adiponectin exerts its functions by binding to adiponectin receptor (AdipoR)1 and AdipoR2. The magnitude of the effects of adiponectin on physiological functions in tissues is directly associated with receptor expression levels (13). AdipoR1 is expressed significantly higher in osteoclasts compared with AdipoR2, suggesting that AdipoR1 has a higher affinity for this receptor isoform (14). Cell growth and metabolism also are regulated by mechanistic target of rapamycin kinase (mTOR), which integrates nutrient, energy and oxygen level information. Previous studies have revealed that the mTOR pathway may be involved in the generation of osteoclasts and affect their bone resorption function (15) Walker et al (16) reported that adiponectin absence coincided with active AMPK/mTOR signaling in adiponectin knockout hepatocellular carcinoma cells, which indicates that mTOR lies downstream of adiponectin. However, it remains unclear how AdipoR1, mTOR and its downstream effector molecule eukaryotic translation initiation factor 4E-binding protein (4EBP1) are involved in the effect of adiponectin on the differentiation and maturation of osteoclasts in patients with MM. To study this question, flow cytometry was used to detect the expression of AdipoR1 on the surface of osteoclast precursor cells and the phosphorylation of mTOR and 4EBP1.

Materials and methods

Study subjects. Subjects were recruited from the Hematology Department of Tianjin Medical University General Hospital (Tianjin, China). The present study was ethically approved by the Ethics Committee of the Tianjin Medical University. Written informed consent was obtained from all patients for the publication of this report and any accompanying images. Bone marrow and peripheral blood were collected from 32 newly diagnosed patients with MM (including 24 men and 15 women; median age, 56 years; range, 46-72 years), according to the International Myeloma Working Group (17). Peripheral blood from normal controls were collected from 20 women; median age, 50 years; range, 25-70 years), according to the International Myeloma Workgroup (18). The diagnoses of patients with MM were made according to the International Myeloma Workgroup criteria. X-ray images were used to stage disease prior to treatment. Bone disease was graded as follows: Stage A, no osteolytic lesions or osteoporosis alone; stage B, one to three osteolytic lesions; and stage C, more than three osteolytic lesions and/or a pathological fracture (18). Patients with MM were classified using the International Staging System (ISS). ISS stage I was defined as serum beta-2-microglobulin levels <3.5 mg/l and serum albumin levels >3.5 g/dl. ISS stage III was defined as serum beta-2-microglobulin level >3.5 mg/l, independent from serum albumin level. ISS stage II included patients who did not fulfill the criteria for stages I and III (19).

Enzyme-linked immunosorbent assay (ELISA). Serum and bone marrow were collected prior to treatment. Sera were centrifuged at 1,000 x g for 10 min at room temperature and then stored at -80°C until use. Concentrations of visfatin, leptin and adiponectin were measured using commercially available ELISA kits (adiponectin (cat. no. SEA605Hu), visfatin (cat. no. SEA638Hu) and leptin (cat. no. SEA084Hu) (all Usn Life Sciences, Inc., Wuhan, China) according to the manufacturer’s protocol.

Electrochemiluminescence immunoassay. The serum levels of carboxy-terminal cross-linking telopeptide of type I collagen (CTX), osteocalcin (OCN) and procollagen I amino-terminal pro-peptide (PINP) were analyzed using kits (cat. no. COBASE601) from Roche Diagnostics GmbH (Mannheim, Germany) according to the manufacturer’s protocol.

Determination and identification of osteoclasts. In brief, BMMNCs were isolated from patients with MM and cultured in α-Minimum Essential Medium containing 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 ng/ml M-CSF (Miltenyi Biotec, Inc., Cambridge, MA, USA) and 100 ng/ml RANKL (Miltenyi Biotec, Inc.). Recombinant human full-length adiponectin (PeproTech, Inc., Rocky Hill, NJ, USA) was dissolved in water at a concentration of 1.0 mg/ml and then diluted with 0.1% bovine serum albumin to a final concentration of 0.1 mg/ml. It then was stored at -20 to -80°C until use. BMMNCs were cultured for 14 days in the presence of 10 μg/ml adiponectin, following which the osteoclasts were identified using TRAP staining at 37°C for 5 min using a commercial TRAP staining kit (Sigma-Aldrich). The number of TRAP-positive multinuclear (>3 nuclei) cells in each well were counted.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol reagent (Tiangen Biotech Co., Ltd., Beijing, China). The quality of RNA samples was assessed spectrophotometrically (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Finally, the extracted RNA was dissolved in 20 μl diethylpyrocarbonate-treated water and cDNA was synthesized using a TIANScript RT kit (Tiangen Biotech co., Ltd.,) according to the manufacturer’s protocol: DNA removal reaction at 42°C for 3 min, RT reaction at 42°C for 15 min, then 95°C for 3 min. RNA levels were quantified by RT-qPCR using a Bio-Rad iQ5 Real-time system (Bio-Rad Laboratories, Inc.) and the thermocycling conditions were as follows: 95°C for 30 sec, 95°C for 3 sec, then annealing temperature for 30 sec, with a total of 45 cycles. The levels of RNA were determined using the 2^-ΔΔcq method using β-actin as a control (20). SYBR Green (Tiangen Biotech Co., Ltd.) was used as a double-strand DNA-specific dye. Primers sequences are presented in Table I.

Flow cytometric analysis. Flow cytometric analysis was conducted using a CytoFlex flow cytometer and CytExpert Pro analysis software 2.0 (Beckman Coulter, Inc., Brea, CA, USA). The side scatter area represent the relative measures of complexity (21). According to a previous study by Sorensen et al (22), CD14 is the specific marker of the osteoclasts precursor derived from CD14+ monocytes cultured with M-CSF and RANKL. Thus, CD14-positive cells are osteoclasts precursor cells. To detect AdipoR1 expression, osteoclast precursor cells were labeled with anti-CD14 (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA; cat. no. 562691) and anti-AdipoR1 (Abcam; cat. no. ab126611) for 15 min at room temperature. Phosphorylation of mTOR and 4EBP1 was
determined as follows: Anti-cd14 antibodies (BD Pharmingen; BD Biosciences; cat. no. 562691) were incubated with cells at room temperature for 15 min. Cells were washed with phosphate buffered saline, then fixed at room temperature for 5 min and permeabilized for 30 min using the Fixation/Permeabilization Solution kit (BD Biosciences) according to the manufacturer's protocol. mTOR signaling pathway-specific agonist (MHY1485) was added (10 µM; 4 h) at 37˚C after 14 days in the presence of 10 µg/ml adiponectin to further determine whether the effects of adiponectin may be reversed. Anti-mTOR (BD Phosflow; BD Biosciences; cat. no. 563489) or anti-4EBP1 antibody (BD Phosflow; BD Biosciences; cat. no. 560285) then were added for 20 min at room temperature.

Statistical analysis. SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All results are expressed as the mean ± SD, median and quartile range. An unpaired Student’s t-test and one-way analysis of variance with the LSD post hoc test were used to analyze the significance of differences between groups. A non-parametric test (Mann-Whitney U test) was used if the data were not normally distributed. The correlation between visfatin, leptin and adiponectin and CTX was performed using a Pearson's test. A Spearman's test was used to determine the correlation between adiponectin, leptin or adiponectin, and OCN or PINP. P<0.05 was considered to indicate a statistically significant difference.

Results

Levels of adiponectin are decreased in newly diagnosed patients with MM. Clinical characteristics of the patients are presented in Table II. Adiponectin levels in the serum from patients with MM were significantly lower compared with in normal controls (12.37±3.13 ng/ml vs. 13.80±0.95 ng/ml; P=0.045). Visfatin levels were significantly higher in the serum from patients with MM compared with the control (102.76±90.41 ng/ml vs. 22.55±21.41 ng/ml; P<0.001). There was no significant difference between the level of leptin in patients with MM and the normal controls (Table II; Fig. 1).

Level of adiponectin is associated with ISS and bone disease stage in MM. The present study compared the levels of adiponectin according to the stage of ISS and bone disease. The serum levels of adiponectin in patients with stage I/II MM was significantly higher compared with in patients with stage III MM (P<0.05; Table III). However, the serum and bone marrow levels of adiponectin in MBD stage A were significantly higher compared with those in stages B or C (P<0.05; Table IV). No significant differences in visfatin or leptin levels were correlated with stage of ISS or bone disease (Tables III and IV).

OCN is positively correlated and CTX is negatively correlated with the level of adiponectin in patients with MM. The present study determined the correlation between serum levels of visfatin, leptin and adiponectin and measures of MM load (β2-microglobulin, plasma cell percentage in bone marrow, serum creatinine and LDH) or serum markers of bone disease (OCN, CTX and PINP). Adiponectin levels significantly correlated negatively with CTX (r=-0.339, P<0.05). Positive significant correlations were identified for OCN (r=0.394, P<0.05; Table V and Fig. 2). The levels of visfatin and leptin were not significantly correlated with MM load or serum markers.

Adiponectin inhibits osteoclast differentiation and maturation. BMMCs were cultured with RANKL and M-CSF for
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14 days, following which TRAP staining was performed. Osteoclasts were observed in each of the groups, but the number of osteoclasts in the adiponectin group were significantly lower compared with that in the control group (P<0.05; Fig. 3). RT-qPCR was also performed to determine the mRNA expression levels of the osteoclast specific factors RANKL, OSCAR, TRAP and Cathepsin K. Expression levels for all factors were significantly lower in the adiponectin group compared with in the control group (P<0.05; Fig. 4).

Adiponectin upregulates AdipoR1 expression on osteoclast precursors. To further investigate the mechanism of action of adiponectin on the differentiation and maturation of MM osteoclasts, the levels of the cell surface expression of AdipoR1 on osteoclast precursor cells (CD14+ cells) was determined using flow cytometry. The levels of AdipoR1 in the adiponectin group were significantly higher compared with in the control group (P<0.05; Fig. 5).

Adiponectin downregulates the phosphorylation of mTOR and 4EBP1. The combination of RANK and RANKL have been revealed to activate the mTOR pathway, which serves an important function in the differentiation and maturation of osteoclasts (23). To determine the effect of adiponectin on this pathway, the phosphorylation of mTOR and its downstream signaling molecule 4EBP1 were measured. The

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**Table II. Profiles of patients with MM enrolled in the present study.**

| Group         | Patient (n) | Male | Female | Age (years), median (range) | ISS | Type of MM | Visfatin (ng/ml, x±s) | Leptin (ng/ml, x±s) | Adiponectin (ng/ml, x±s) |
|---------------|-------------|------|--------|----------------------------|-----|------------|----------------------|---------------------|------------------------|
| MM (serum)   | 39          | 24   | 15     | 63 (47-83)                 | I/II| IgG        | 102.76±90.41*         | 0.21±0.11           | 12.37±3.13*           |
| Control      | 20          | 11   | 9      | 67 (57-75)                 |     | IgA κ      | 22.55±21.41           | 0.21±0.13           | 13.80±0.95             |

*P<0.05 vs. the control group. MM, multiple myeloma; Ig, immunoglobulin; ISS, International Staging System; x±s, mean ± SD.

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**Table III. Levels of visfatin, leptin and adiponectin in patients with different ISS phase multiple myeloma.**

| ISS | Patient (n) | Visfatin (ng/ml) | Leptin (ng/ml) | Adiponectin (ng/ml) |
|-----|-------------|------------------|----------------|---------------------|
| I/II| 8           | 60.66±54.68      | 0.20±0.05      | 14.47±1.29*         |
| III | 31          | 113.62±95.19     | 0.21±0.12      | 11.83±3.24          |

*P<0.05 vs. the III group. Data presented as the mean ± SD. ISS, International Staging System.

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**Table IV. Levels of visfatin, leptin and adiponectin in different multiple myeloma bone disease stages.**

| Bone disease | Patient (n) | Visfatin (ng/ml) | Leptin (ng/ml) | Adiponectin (ng/ml) |
|--------------|-------------|------------------|----------------|---------------------|
| Stage A      | 8           | 55.38±49.57      | 0.17±0.05      | 14.36±2.32*         |
| Stage B/C    | 26          | 114.98±95.01     | 0.22±0.12      | 11.86±3.13          |

*P<0.05 vs. the stage B/C group. Data presented as the mean ± SD.

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**Figure 1. Visfatin, leptin, and adiponectin levels in newly diagnosed patients with MM.** *P<0.05 with comparisons shown by lines. MM, multiple myeloma.
phosphorylation levels of mTOR and 4EBP1 were significantly lower in the adiponectin group compared with in the control group (P<0.05; Fig. 6). Additionally, a mTOR signaling pathway-specific agonist (MHY1485) was used to further determine whether the effects of adiponectin may be reversed. The results revealed that the levels of p-mTOR and p-4EBP1 in the adiponectin + MHY1485 group were significantly higher compared with that in the adiponectin alone group (P<0.05; Fig. 6). It revealed that adiponectin may inhibit osteoclast differentiation and maturation via the mTOR pathway.

**Discussion**

Previous studies have revealed that obesity is a risk factor for MM (24) and MBD due to the associated increase in the release of adipocytokines (25). The present study studied the association between markers of MBD and the adipocytokines visfatin, leptin and adiponectin in patients with MM. Visfatin is known to facilitate the proliferation of pre-B cells and digestive system neoplasms (5,18). Another form of visfatin, nicotinamide phosphoribosyl transferase, is indispensable for myeloma cell growth and osteoclast activity, though its effect on MM cells requires further investigation (25). The present study revealed that the levels of visfatin were significantly higher in sera from patients with MM compared with sera from normal controls (P<0.05) but that there was no correlation between visfatin levels and the severity of MBD.
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Figure 4. mRNA levels of the osteoclast-specific factors RANKL, OSCAR, TRAP and Cathepsin K. Expression levels of mRNA are presented for the control and adiponectin-treated group for (A) RANKL, (B) OSCAR, (C) TRAP and (D) Cathepsin K. *p<0.05 with comparisons shown by lines. RANKL, receptor activator of nuclear factor-κB ligand; OSCAR, osteoclast associated Ig-like receptor; TRAP, tartrate-resistant acid phosphatase.

Figure 5. Adiponectin upregulates the AdipoR1 expression level. AdipoR1 levels in (A) the control group and (B) the adiponectin-treated group. (C) Quantification of the AdipoR1 levels in the two groups (26.21±4.27 vs. 29.86±6.23%). *p<0.05 with comparisons shown by lines. AdipoR1, adiponectin receptor 1.
Reseland et al. (26) demonstrated that plasma concentration of leptin was significantly higher in newly diagnosed patients compared with a healthy control group. While the present study demonstrated that serum leptin levels in 39 patients with MM were not significantly different compared with those in normal controls, and no correlation existed between leptin levels and MM load or MBD. Further investigation is required to determine if the levels of leptin may affect MM cells.

Adiponectin is secreted not only by adipocytes but also by mesenchymal stem cells, osteoclasts and adipose cells in the bone marrow (27). Downregulation of adiponectin may increase the proliferation of MM cells in mouse models of disease (8). Dalamaga et al. (28) demonstrated that lower levels of adiponectin predicted a higher risk of MM. In the present study, adiponectin levels were significantly lower in newly diagnosed patients with MM compared with in controls (P<0.05). Stage III patients had lower levels compared with stage I/II patients. In patients with MBD, adiponectin levels of stage B/C patients were lower compared with those of stage A patients. These results are consistent with previous studies (26,28). The present study observed a trend toward lower adiponectin levels in normal controls compared with those in normal controls, and no correlation existed between leptin levels and MM load or MBD. Further investigation is required to elucidate the underlying mechanism.

Adiponectin activates intracellular signaling through its receptors, AdipoR1 and AdipoR2, and the expression levels of these receptors correlate directly with the magnitude of the ensuing signaling (13). AdipoR1 is expressed at significantly higher levels in osteoclasts compared with AdipoR2, suggesting that AdipoR1 has a higher affinity for this receptor

Adiponectin has positive effects on insulin sensitivity, inflammation, oxidative stress and tumor growth (33,34). In the present study, osteoclasts were successfully induced by RANKL and M-CSF subsequent to extracting mononuclear cells from patients with MM. TRAP staining revealed that the number of osteoclasts in the adiponectin group was lower compared with that in the control group. In addition, adiponectin decreased the mRNA levels of RANKL, OSCAR, TRAP and Cathepsin K, indicating that adiponectin may inhibit osteoclast differentiation and maturation in MM. The limitation of the present study is that only RT-qPCR was performed to assess the expression of osteoclast-specific factors RANKL, OSCAR, TRAP and Cathepsin K. Therefore, it is unclear whether the protein levels of these factors change in a similar manner, as western blot analysis was not performed due to the objective difficulty that the samples from the patients with MM were limited.
isoform (14). Thus, the present study did not measure the expression of AdipoR2. To examine how adiponectin affects osteoclast differentiation, the present study examined the effect of adiponectin on the cell surface expression of AdipoR1 on osteoclast precursors. The results revealed that the expression of AdipoR1 increased following adiponectin treatment, suggesting that adiponectin affects the differentiation and maturation of osteoclasts by increasing the expression of AdipoR1.

The molecular pathways downstream of AdipoR1 have not yet been fully elucidated. However, a number of studies have revealed that the mTOR signaling pathway, which is also associated with cell growth, is involved (35,36). mTOR serves an important function in regulating cell proliferation, growth, differentiation, migration and survival (35-37). Phosphorylated AMPK inhibits mTOR and may be activated by phosphorylated mTOR. The phosphoinositide-3-kinase/protein kinase B/mTOR pathway serves an important function in regulating bone remodeling, which negatively regulates bone mineralization (35-38) in vitro and promotes osteoclastogenesis (39-41). Previous studies have revealed that the inhibition of mTOR signaling may reduce bone loss in patients with rheumatoid arthritis, multiple myeloma or neurofibromatosis (42-44). Adiponectin also may inhibit the growth of colorectal cancer cells by inhibiting the mTOR cell pathway (45). In the present study, it was revealed that adiponectin upregulates the expression of AdipoR1 on osteoclast precursor cells and inhibits the phosphorylation of mTOR and 4EBP1. Additionally, a mTOR signaling pathway-specific agonist (MHY1485) was used to further determine that the effects of adiponectin may be reversed. It revealed that adiponectin may inhibit osteoclast differentiation and maturation via the mTOR pathway. Therefore, adiponectin inhibits the differentiation of osteoclasts in MM and this effect may be mediated by the inhibition of mTOR signal transduction following the binding of adiponectin by its receptor. However, the specific molecular mechanism requires further investigation.

Adiponectin may serve a crucial function in MBD by inhibiting the differentiation and maturation of osteoclasts in patients with MM. This effect may be mediated by increasing the expression of AdipoR1 on the surface of osteoclast precursor cells through mTOR signaling.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

ZL, HL and RF designed this research. ZL and YL performed the majority of the experiments, analyzed the data, drew the figures and drafted this manuscript. YW, RX and FM helped with cell culture, reverse transcription-quantitative PCR and the flow cytometry. CX performed the cell culture. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was ethically approved by the Ethics Committee of the Tianjin Medical University (Tianjin, China). Written informed consent was obtained from the patients.

Patient consent for publication

Written informed consent was obtained from the patients for the publication of this report and any accompanying images.

Competing interests

The authors declare that they have no competing interests.

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