

IGFBP-1 Promotes RANKL-Induced Osteoclastogenesis via the ROS-Mediated Apoptotic Pathway

Xiaolin Zhu

Qingdao Municipal Hospital Group <https://orcid.org/0000-0002-0452-3031>

Nailong Yang (✉ nailongy@163.com)

The Affiliated Hospital of Qingdao University, Qingdao, Shandong <https://orcid.org/0000-0002-4626-8219>

Research

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Abstract

Background: Osteoporosis acts as a type of metabolic disease which deteriorates osteopenia and bone microstructure. Osteoclasts serve as the major effector cells which have the function of degrading the bone matrix, and the abnormal function of osteoclasts cause the occurrence of osteoporosis. It has been identified that hepatokine insulin-like growth factor binding protein 1 (IGFBP-1) could promote receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclastogenesis in vitro. Meanwhile, IGFBP-1 is an BAK-binding regulator of apoptosis in hepatocytes and accumulation of reactive oxygen species (ROS) may trigger apoptosis via many apoptotic signaling pathways. So we speculated that IGFBP-1 may affect osteoclastogenesis, at least in part, via the ROS-mediated apoptosis pathway.

Methods: RAW264.7 (mouse macrophage cells) were stimulated with IGFBP-1 with or without RANKL. Osteoclastogenesis was detected by tartrate-resistant acid phosphatase (TRAP) staining and *quantitative real-time polymerase chain reaction (PCR)*. Then flow cytometry (FCM) was adopted to measure the apoptotic rate presented by the TRAP-positive multinucleated osteoclasts. Meanwhile the expression of different proteins related to apoptosis and the ROS levels was detected in RAW264.7 cells of different groups.

Results: IGFBP-1 could promote RANKL-induced osteoclastogenesis in vitro and obviously attenuate the accumulation of ROS. In addition, IGFBP-1 treatment could inhibit the cell apoptosis and devitalize caspase-3 and Bax/Bcl-2 ratio in the process of RANKL-induced osteoclastogenesis.

Conclusions: IGFBP-1 could promote the bone loss induced by RANKL via apoptosis inhibition of osteoclasts by down-regulating the accumulation of ROS and the apoptosis pathway dependent on mitochondria. Hence, IGFBP-1 could be a novel therapeutic candidate for osteoporosis treatment.

Background

The balance between bone-forming osteoblasts and bone-resorbing osteoclasts helps to preserve the integrity of skeleton and bone. The osteoclast takes charge of the removal of the organic component and inorganic component of bone. Despite the small proportion it occupies in bone cells, osteoclast can help to maintain the normal skeletal strength obviously relying on various activities. Nevertheless, the formation and resorption of osteoclast bring in pathological stimulation, thereby causing many diseases including postmenopausal osteoporosis, inflammatory arthritis, cancer bone metastasis, etc (1). For investigating the differentiation and activity of osteoclast, raw 264.7 has become a valuable tool relying on its expression of RANK and differentiation to the osteoclast affected by RANKL (2). A series of endocrine hormones together with metabolic signals can regulate the process. New understanding of the regulation of osteoclastogenesis and bone resorption will contribute to better treatment method as well as effective insights into the pathology of disease (1).

The insulin-like growth factor-1 (IGF-1) system remarkably affects the bone remodeling. IGFBP-1 is one of six IGFBPs, which bind to and regulate bioavailability of IGF-1. Apart from decreasing bone formation by

inhibition of IGF-1 actions, IGFBP-1 can also promote bone resorption by stimulating osteoclast differentiation, by IGF-1-independent mechanisms (3). In brief, IGFBP-1 is a pro-osteoclastogenic liver hormone induced by FGF21, which binds to its receptor Integrin β 1 on the osteoclast precursors, thereby playing its function, so as to potentiate the Erk-phosphorylation as well as NFATc1 activation stimulated by RANKL (3). Clinical finding indicates that high IGFBP-1 was related to the increasing fracture risk, unrelated to IGF-1 or BMI but partially mediated by the BDM, which supports the abovementioned effect of IGFBP-1 (4). In addition, when compared with control subjects of age and sex, the concentration of IGFBP-1 for osteoporosis patients was four times higher (5). Meanwhile, IGFBP-1 is an BAK-binding regulator of apoptosis in hepatocytes (6). RGD peptides block the integrin interaction with the bone matrix, thereby accelerating the apoptosis of osteoclast (7). So it can be speculated that IGFBP-1 may affect osteoclastogenesis, at least in part, via the apoptosis pathway.

Apoptosis, as an important mechanism, is applied by mechanism to removing unnecessary cells for precisely controlling the development and function of organ. Accumulation of ROS may trigger apoptosis via many apoptotic signaling pathways, particularly apoptotic pathways dependent of mitochondria. Abovementioned apoptotic events dependent of mitochondria are under the mediation of Bcl-2 family of proteins which determine the destiny of cells through anti-apoptosis (Bcl-2, Bcl-X, Bcl-w, etc.) as well as pro-apoptosis (Bax, Bad, Bak, etc.) members (8). Based on recent finding about animals and clinical studies, there is an obvious association between the oxidative stress and ROS and the pathogenesis regarding bone loss related to age (9, 10). ROS is able to damage the mitochondrial ultrastructure as well as destroy its function, thereby activating the caspase-3 via different molecular cascade reactions and activate mitochondrial apoptotic pathway. Therefore, researchers are suggested to focus on the relation between apoptosis induced by ROS and osteoclastogenesis induced by IGFBP-1.

As found in the study, IGFBP-1 could promote RANKL-induced osteoclastogenesis via evaluating IGFBP-1 on TRAP staining as well as the expression of a lot of marker genes. IGFBP-1 could inhibit cell apoptosis via reducing intracellular ROS generation and inhibiting signaling pathway dependent of mitochondria. All these could inhibit the formation and function of mature osteoclast.

Materials And Methods

Materials

Dulbecco's modified eagle medium (DMEM) (11960-044), fetal bovine serum (FBS), Glutamax (35050-061) as well as Sodium Pyruvate (11360-070) were provided by Gibco (Invitrogen). Recombinant mouse IGFBP-1 Protein (1588-B1) and Recombinant mouse RANKL (462-TEC) were provided by R&D Systems.

Cell culture

RAW264.7 were provided by American Type Culture Collection (ATCC, USA) and cultured in complete medium containing 88%DMEM, 10% FBS, 1% Glutamax and 1% Sodium Pyruvate at 37°C in a humidified atmosphere which contained 5% CO₂. In brief, RAW264.7 were randomly divided into 4 groups: complete

medium-treated (RANKL-), complete medium and IGFBP-1 (50 ng/ml)-treated (IGFBP-1), complete medium and RANKL (50 ng/ml) -treated (RANKL+), complete medium, IGFBP-1 (50 ng/ml) and RANKL (50 ng/ml)-treated groups (R + IGFBP-1).

Osteoclast differentiation and staining

We seeded RAW264.7 on a 96-well plate at a density of 1×10^3 cells each well and used 50 ng/ml RANKL to culture them with the medium changed each two days. On day 5, mature multinucleated osteoclasts were fixed and stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma-Aldrich), based on manufacturer's protocol. TRAP + cells containing over 3 nuclei were termed as the mature osteoclasts, and counted in each well.

Intracellular ROS detection

ROS assay kit (Solarbio, China) with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) helped to determine the ROS levels in these cells. RAW264.7 were seeded on a 6-well plate at a density of 2×10^5 cells each well and treated by using various reagents. After 72 h treatments, the cells received 20 min of incubation by using $10 \mu\text{M}$ DCFH-DA in cell incubator at 37°C and then washed by using serum-free DMEM media three times. The fluorescence microscopy (OLYMPUS Tokyo, Japan) was used to obtain the images.

Flow cytometry apoptosis analysis

We seeded RAW264.7 on a 6-well plate at a density of 2×10^5 cells each well and used different reagents to treat them. ANNEXIN V-FITC/PI kit (Solarbio) helped to study how IGFBP-1 affects osteoclastogenesis. Following the treatment, cold PBS was used to wash these cells twice. Subsequently, cells were digested in 0.25% trypsin at 37°C , followed by being resuspended in the binding buffer together with the annexin V-FITC/PI staining based on manufacturer's instruction. A FACScan flow cytometer was employed to analyze these samples.

RNA extraction and quantitative real-time PCR

We seeded RAW264.7 on a 6-well plate at a density of 2×10^5 cells each well and used different reagents to treat them for five 5 days. TRIzol reagent (Sigma-Aldrich Chemical Co.) was used to extract the total RNA. The reverse transcriptase kit (Takara) helped to synthesize the complementary DNA (cDNA) from a certain amount of total RNA (1 ug), followed by amplifying the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The real-time quantitative polymerase chain reaction (RT-PCT) was carried out at 95°C for 30 s, and then 40 cycles (95°C for 5 s and 60°C for 30 s). The experiment was performed three times for confirming reproducibility and the $2^{-\Delta\Delta\text{Ct}}$ method was applied to analyzing the relative expression levels of genes. Sequences of primers for the reference gene (GADPH) and interested genes are listed as follows:

GAPDH: F-5'-TGCACCACCAACTGCTTAGC-3',

R-5'-GGCATGGACTGTGGTCATGAG-3';

TRAP: F-5'-CAGCGACAAGAGGTTCCA-3',

R-5'-GGTATGTGCTGGCTGGAA-3';

CTSK: F-5'-TGTGACCGTGATAATGTGAA-3',

R-5'-GCAGGCGTTGTTCTTATTC-3';

MMP-9: F-5'-AACTCACACGACATCTTC-3',

R-5'-CACCTTGTTACCTCATT-3';

Western blot analysis

We seeded the RAW264.7 on a 6-well plate at a density of 2×10^5 cells each well and using different agents to treat them for four days. The radioimmunoprecipitation assay buffer(RIPA; Millipore, MA) which contained protease and phosphatase inhibitors was used to extract total protein from the osteoclasts. The BCA protein assay kit helped to analyze the concentration of the protein. 10% SDS-PAGE gel electrophoresis was used to separate 20 ug of protein in total, which were subsequently transferred to a polyvinylidene fluoride(PVDF) membrane. The membranes were blocked with 5% non-fat milk was used to block these membranes for two hours, followed by overnight incubation at 4°C by using primary antibodies. Furthermore, the membranes received 1 h of incubation with secondary antibodies and Tris-buffered saline with Tween(TBST) was used to wash them. An Odyssey imaging system(Li-Cor, Lincoln, NE) was applied to detecting the flurescent signals.

Statistical analyses

Experiments were performed repeatedly for no less than 3 times. SPSS 23.0 was used to analyze all data. A one-way analysis of variation (ANOVA) together the Student-Newman-Keuls test were performed for the multiple comparisons. All data are expressed as the mean \pm SD. *P < 0.05 and **P < 0.01 were considered as statistically significant.

Results

IGFBP-1 promotes RANKL-induced osteoclastogenesis

RAW264.7 helped to carry out an osteoclastogenesis assay for determining whether the IGFBP-1 can facilitate the osteoclast formation induced by RANKL. RANKL (50 ng/ml), IGFBP-1 (50 ng/ml) or both were used to treat these cells for 5 days, followed by the TRAP staining for checking how IGFBP-1 affects RANKL-induced osteoclastogenesis (Fig. 1A). TRAP-positive cells with over 3 nuclei were counted as the osteoclasts. Based on quantification analysis, IGFBP-1 could increase RANKL-induced osteoclastogenesis (Fig. 1B). For substantiating above finding, the expression of the specific osteoclast markers was further

measured. The expression of TRAP, CTSK and MMP-9 were all up-regulated at mRNA level with the treatment of IGFBP-1 (Fig. 1C). On that account, IGFBP-1 could promote RANKL-induced osteoclastogenesis.

IGFBP-1 inhibited cell apoptosis in the process of RANKL-induced osteoclastogenesis

For exploring the potential influencing mechanism that IGFBP-1 promotes the osteoclastogenesis induced by RANKL, flow cytometry (FCM) was adopted to measure the apoptotic rate presented by the TRAP-positive multinucleated osteoclasts. ANNEXIN V-FITC/PI staining revealed that RANKL treatment alone showed the higher cell apoptosis rate in early and late stages in comparison with the control groups (Fig. 2A and B). However, IGFBP-1 effectively protected osteoclasts against RANKL-induced apoptosis (Fig. 2A and B).

IGFBP-1 inhibited apoptosis in osteoclastogenesis induced by RANKL relying on mitochondria-dependent pathway

For figuring out the potential influencing mechanism that IGFBP-1 promotes the osteoclastogenesis induced by RANKL, western blot analysis was applied to the detection of the expression of proteins related to apoptosis in RAW264.7 of different groups after four days of treatment (Fig. 3A). Figure 3B illustrates the obvious reduction of the protein expression of Bax and cleaved caspase-3, and the obvious increase in the Bcl-2 expression in groups under the treatment of both RANKL and IGFBP-1. The results indicated the association between the suppression of IGFBP-1 on apoptosis in the process of RANKL-induced osteoclastogenesis and the mitochondrial dysfunction.

IGFBP-1 protected apoptosis induced by RANKL via modulating the intracellular ROS generation

Figure 4. showed that RANKL can remarkably enhance the level of cellular ROS, while IGFBP-1 can inhibit the ROS generation induced by RANKL in an effective manner. Therefore, the study directly proves that IGFBP-1 prevents apoptosis induced by RANKL in osteoclasts via weakening ROS generation.

Discussion

Given the clinical association between the chronic liver diseases and the osteoporosis, people for the long term speculate that certain liver-derived preteins can regulate the bone mass. However, IGFBP-1 is a liver-specific factor, which had the highest mRNA in liver compared with other tissues (over 100-fold), proving its function as a hepatokine (3). Patients with cirrhosis (11) and bone loss (4) exhibited a higher circulating level of IGFBP1. Based on these, it has been identified that IGFBP-1 is a hormone which is produced in liver and facilitates the osteoclastogenesis as well as bone resorption (3). Therefore, it is elusive to prove the direct evidence of a "liver-bone axis". In addition, hepatic IGFBP-1 protein localized to mitochondria is a prosurvival factor which prevents liver from being affected by apoptosis (6). So we

speculate that IGFBP-1 may affect osteoclast differentiation through the mitochondria-dependent apoptotic pathway. Based on our results, IGFBP-1 has been proven to promote osteoclastogenesis through ROS-mediated apoptosis pathway. According to these findings, IGFBP-1 may potentially be a target of molecular targeted therapy in conditions such as osteoporosis. In clinical practice, an anti-IGFBP-1 drug may prevent osteoporosis in patients with high IGFBP-1 protein expression.

Two different signaling pathways can control the apoptosis, one is initiated by the death receptors and another is regulated by the Bcl-2 family proteins. Many animal models with genetic modification have proved the importance of osteoclast apoptosis in the bone disorders. As the osteoclast apoptosis decreased, the bone loss was increased in general, for example, after the ovariectomy resulted from estrogen deficiency (12, 13). As the osteoclast apoptosis decreased in the inflamed joints, the bisphosphonate therapy efficacy in the prevention of local bone loss in rheumatoid arthritis patients was reduced partially, in comparison with osteoporosis patients (14). It seems that osteoclast apoptosis can be stimulated by the extrinsic signals which have the function of activating the death receptors on osteoclast surface (15) or the intrinsic signals which have the function of disrupting mitochondrial membrane integrity, thus inducing the apoptosis dependent of caspase (16). Previous literature (17, 18) have well confirmed the function of caspase-3 in the osteoclast apoptosis and we have demonstrated IGFBP-1 treatment could lead to the activation of caspase-3 and then the cell death.

Oxidative stress occurs due to the overproduction of ROS instead of being balanced by sufficient antioxidants. A lot of diseases are associated with the oxidative stress such as bone diseases and thereinto, osteoporosis is one of the most significant. Recent studies implicate the accumulated reactive oxygen species (ROS) and enhanced oxidative stress as the essential factors in osteoporosis related to age (19–21). Osteoclasts exhibit a strong sensitivity to the oxidative stress, which can suppress osteogenesis (22–25). ROS at a low level is likely to stimulate the bone resorption of osteoclast (26–28), however, out of a specific threshold, osteoclasts being chronically exposed to increased oxidative stress leads to cytotoxic effects given the enhanced oxidative damage of DNA, lipids and proteins, thereby causing the apoptosis relying on the caspase-dependent pathway (29). The study showed the ability of RANKL to remarkably improve the intracellular ROS level in the process of osteoclastogenesis (Fig. 4.). ROS level saw a downregulation with IGFBP-1 being introduced into the osteoclastogenesis process mediated by RANKL (Fig. 4.). Hence, the apoptosis rate declined as the ROS reduced (Fig. 2A-B). As mitochondria contained a lots of osteoclasts which serve as the main source of ROS, it is found that the intrinsic apoptotic pathway mainly participated in the osteoclast apoptosis induced by ROS (16). A lot of reports have demonstrated the involvement of B cell leukemia/lymphoma 2 (Bcl-2), Bcl-2 associated X protein (BAX) as well as caspase-3 in the apoptosis pathway dependent of mitochondria on cancer (26). So the mitochondrial apoptotic factors such as Bcl-2, BAX and caspase-3 were further investigated. Based on the finding in the study, RANKL treatment coupled with IGFBP-1 imposed an effect in remarkably decreasing the pro-apoptotic protein Bax expression and increasing the anti-apoptotic protein Bcl-2, as a result, the ratio of Bax/Bcl-2 was decreased (Fig. 3A-B). On that account, IGFBP-1 inhibited osteoclast apoptosis in RANKL-induced osteoclastogenesis via ROS decelerating as well as mitochondrial apoptotic pathway.

Conclusion

In summary, IGFBP-1 treatment in the process of RANKL-induced osteoclastogenesis inhibited the cell apoptotic death due to reduced release of ROS, declined caspase-3 and Bax/Bcl-2 ratio. Even though it is necessary to perform more research to figure out the potential mechanism, the finding can contribute to a better understanding of the IGFBP-1 applied to treating osteoporosis and developing novel therapeutic strategies.

Declarations

Acknowledgements

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NY conceived and designed the study. XZ performed the experiments, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

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Figures

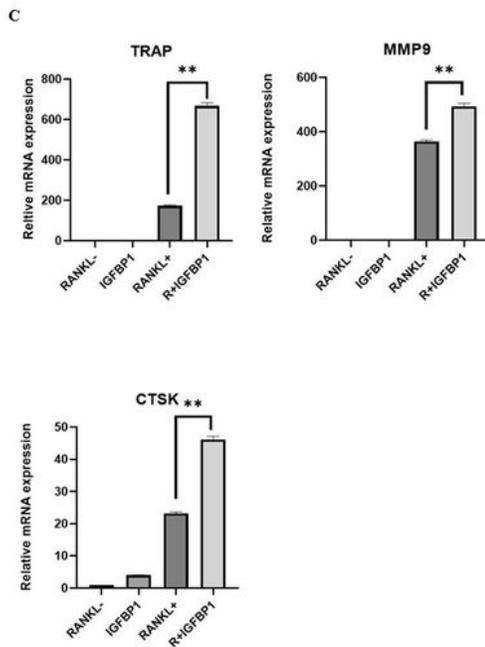
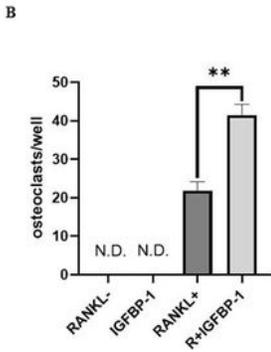
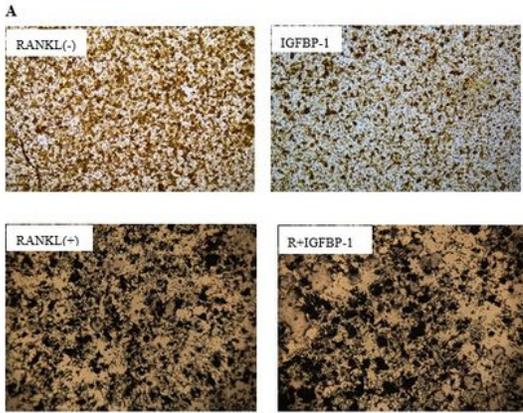


Figure 1

IGFBP-1 promoted RANKL-induced osteoclastogenesis. (A) Representative images presented by RAW264.7 cells stained for TRAP following RANKL(50 ng/ml), IGFBP-1(50 ng/ml) or both for 5 days. (B) Quantification of TRAP(+) cells with over 3 nuclei per well (96-well plate). The data in figures are expressed as the mean±S.D. (C) Relative levels of mRNA expression of TRAP, CTSK and MMP-9 of

RAW264.7 cells in distinct groups on the 5th day. The data in figures are expressed as the mean±S.D. The obvious difference between treatment group and control group is expressed as *P<0.05 or **P<0.01.

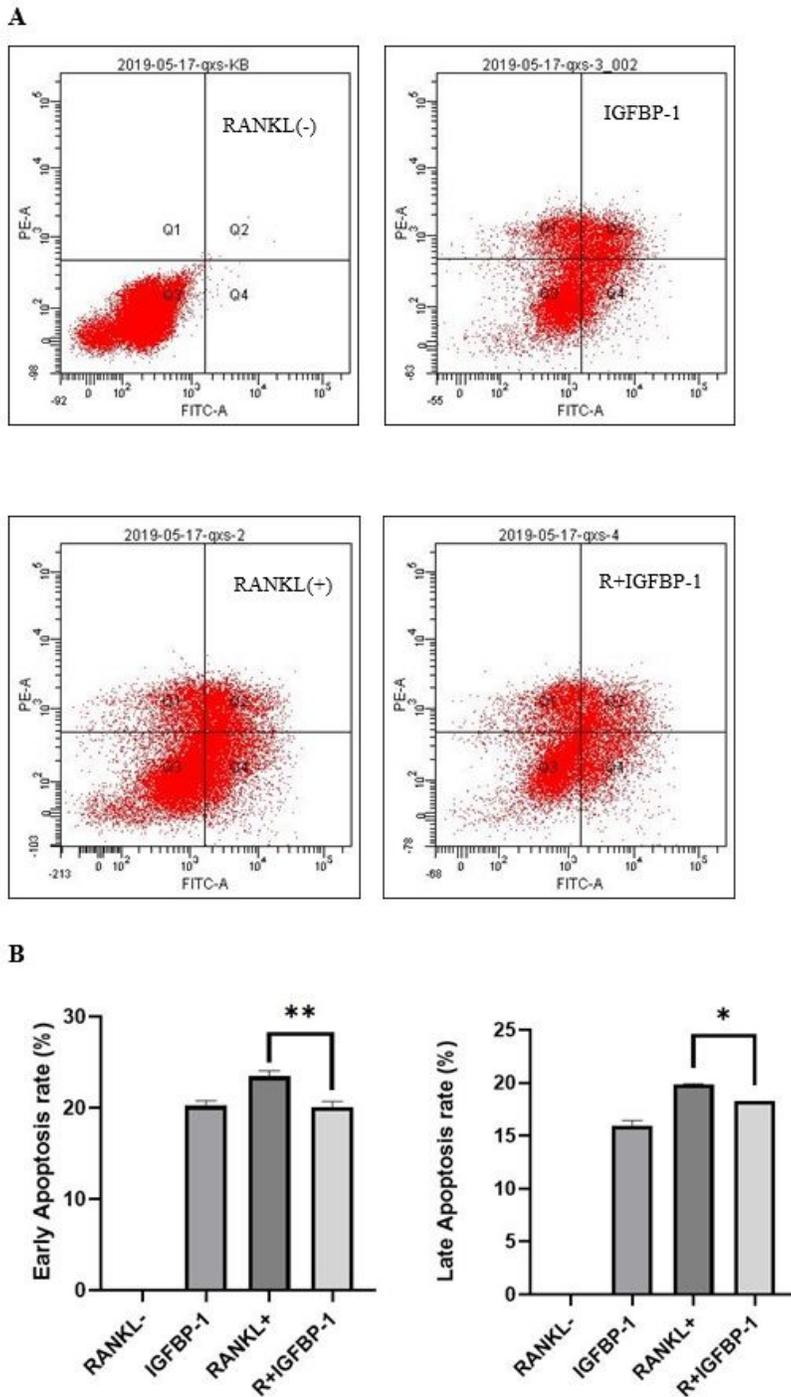


Figure 2

IGFBP-1 suppressed cell apoptosis rate in the process of RANKL-induced osteoclastogenesis. (A) FCM analysis on the cell apoptosis rate exhibited by RAW264.7 under the treatment of RANKL (50 ng/ml), IGFBP-1 (50 ng/ml) or both for three days. (B) Quantification analysis on the cell apoptosis rate in early

and late stages. The data in figures are expressed as the mean±S.D. Obvious differences between treatment group and control group are expressed as *P<0.05 or **P<0.01.

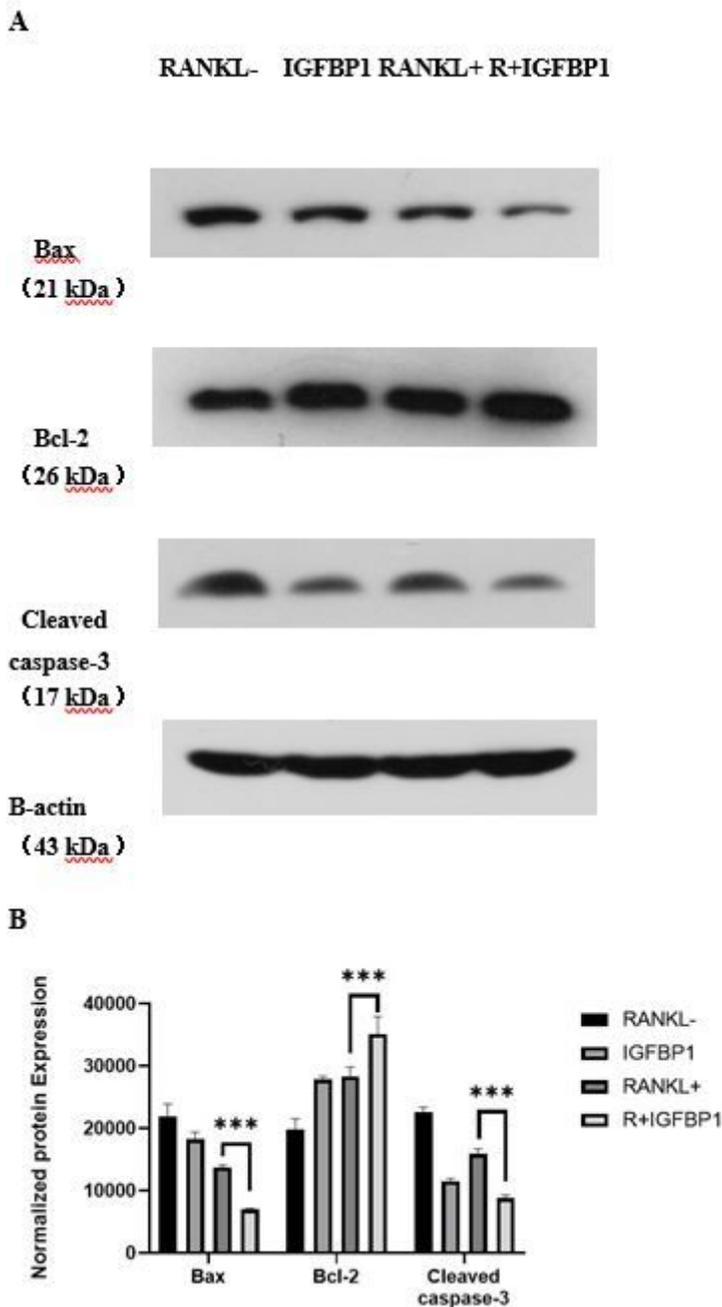


Figure 3

IGFBP-1 suppressed apoptosis in osteoclasts induced by RANKL relying on the mitochondria-dependent pathway. (A) Representative western blot images presented by cleaved caspase-3, Bax, Bcl-2 and β -actin from RAW264.7 under the treatment of RANKL(50 ng/ml), IGFBP-1 (50 ng/ml) or both for three days. (B) Quantification or normalized protein expression intensity presented by caspase-3, Bax and Bcl-2. Data in figures are expressed as the mean±S.D. Obvious differences between treatment group and control group are expressed as *P<0.05, ** P<0.01 or ***P<0.001.

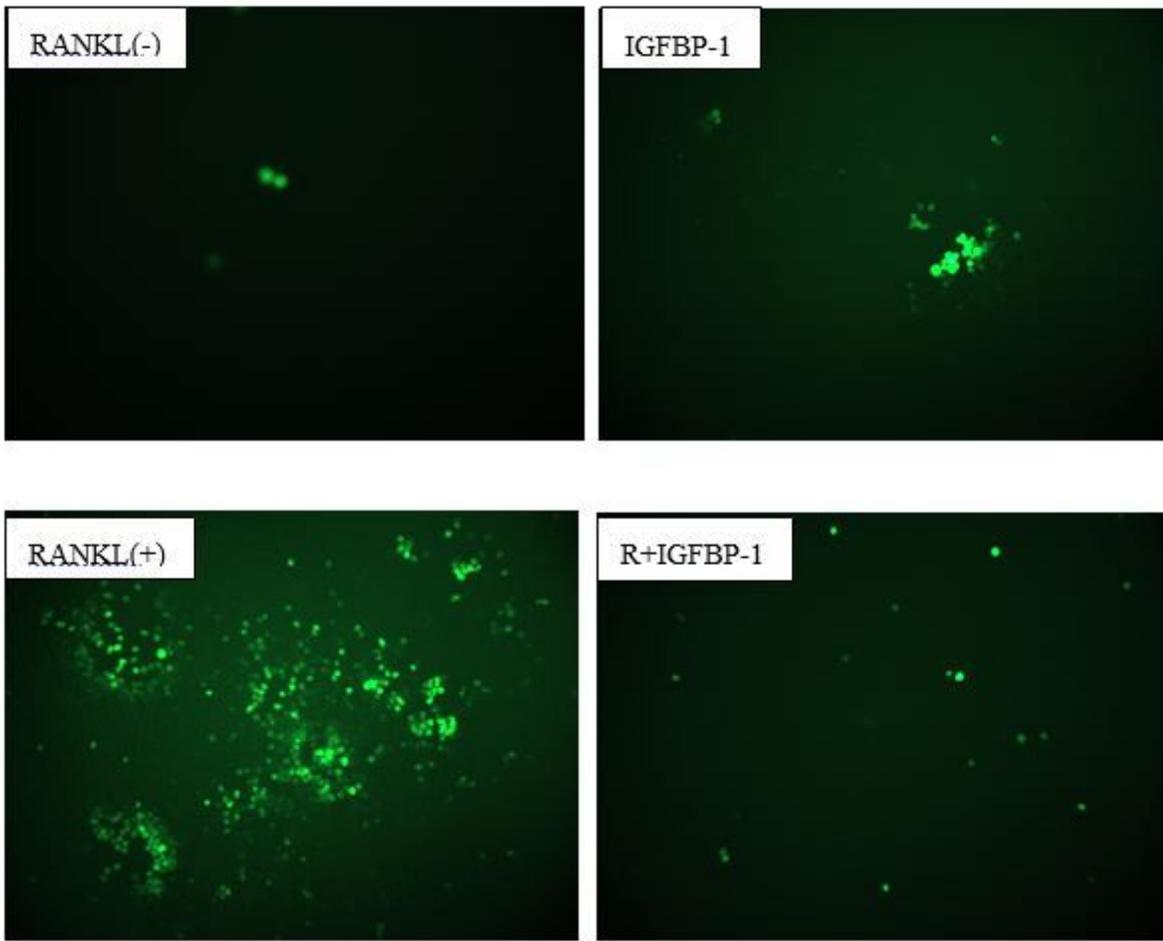


Figure 4

IGFBP1 inhibited intracellular ROS generation during RANKL-induced osteoclastogenesis. ROS generation was evaluated by fluorescence microscopy. Representative images presented by ROS-positive preosteoclasts under the treatment of RANKL(50 ng/ml), IGFBP1(50 ng/ml) or both for five days.