Berberine affects osteosarcoma via downregulating the caspase-1/IL-1β signaling axis

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Abstract. Osteosarcoma is one of the most devastating cancers with associated poor prognosis. Chronic bone inflammation frequently predisposes to tumorigenesis and progression of osteosarcoma. In the tumor inflammatory microenvironment, caspase-1 and its processed cytokines such as interleukin 1β (IL-1β) play an important role in the occurrence and development of cancer. Berberine is an isoquinoline alkaloid extracted from the dry root of *Coptidis Rhizoma*, which has been found to exhibit significant anticancer effects on a wide spectrum of carcinomas including osteosarcoma. However, the mechanisms underlying the anticancer effects of berberine in osteosarcoma remain poorly understood and their elucidation is critical for developing improved therapies. In the present study, we investigated the potential mechanism underlying the anticancer effect of berberine in osteosarcoma. We found that the expression of caspase-1 and its downstream target IL-1β were higher in osteosarcoma cells compared with normal cells both *in vitro* and *in vivo*. Furthermore, administration of berberine is capable of reducing the expression of caspase-1 and IL-1β in osteosarcoma cells and inhibiting the growth of tumor cells. Based on the above, for the first time, we propose the hyposis that berberine could gengerate an anti-osteosarcoma property through downregulating caspase-1/IL-1β inflammatory signaling axis.

Introduction

Osteosarcoma is the most prevalent aggressive malignant bone tumor arising from primitive transformed cells of mesenchymal origin in children and young adults (1). Osteosarcomas tend to occur at the sites of bone growth, presumably due to the proliferation of osteoblastic cells prone to acquire more osteosarcoma mutations which could lead to carcinomatous change of cells (2). In the present study, the treatment therapies of osteosarcoma was mainly performed by surgery combined with chemotherapy due to its relatively resistance to radiotherapy. However, the application of tumor chemotherapy drugs have many adverse effects and tumor cells are prone to acquire drug resistance (3,4). Thus, to find a low toxicity, high efficiency anticancer drugs from natural compounds in the treatment of osteosarcoma has important significance.

The tumor microenvironment is commonly considered as an obligatory and significant component of almost all types of cancer, and the cells infiltrating such microenvironment are a source of inflammatory cytokines. Cytokines like IL-1β plays a key role in regulating inflammation during the development and progression of cancer (5). The IL-1β have pleiotropic effects on various carcinoma cells in the tumor microenvironment, particularly being capable of regulating pro-oncogenic transcription factors STAT3 and NF-κB (6). Therefore, to reveal the IL-1β involved, inflammation related mechanisms are vitally important for the treatment of osteosarcoma.

Caspase-1 is a kind of cysteine protease that proteolytically cleaves other proteins, such as the precursor forms of the inflammatory cytokines IL-1β, into active mature peptides. Consequently, IL-1β activate its downstream NF-κB signaling and thus, enhance the release of inflammatory cytokines (7). Caspase-1 and IL-1β have been shown to play important roles in inflammation, necrosis and pyroptosis, and may function in various developmental stages (8-11). Our preliminary experiments found that the expression of caspase-1 was higher in osteosarcoma tissues than normal bone tissues. However, to the best of our knowledge, the role of caspase-1 and its downstrem target IL-1β in osteosarcoma have not been clarified.

Berberine was derived from traditional Chinese medicinal herbs which have drawn extensive attention towards its antineoplastic effects. It has been reported to suppress growth of a wide variety of tumor cells, including osteosarcoma, breast cancer and gastric carcinoma (12-15). Berberine potently inhibits osteosarcoma growth and metastasis as reported in a previous extensive study and the underlying mechanism is controversial and not that obscure (16,17). Therefore, development of effective therapeutic strategies based on a better understanding of the molecular mechanisms underlying the anti-osteosarcoma property of berberine is urgently needed.

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In the present research, we studied the effects of berberine on osteosarcoma cells *in vivo* and *in vitro*. Our data provided mechanistic insight into the role of berberine in inhibition of osteosarcoma cell growth via downregulating caspase-1/IL-1β inflammatory signaling pathway.

Materials and methods

Cell culture and treatment. Human Saos-2 and MG-63 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Berberine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) in an atmosphere of 95% humidified air and 5% CO_2 at 37˚C. Cells were investigated within 8 h of harvest. To detect the effects of berberine on osteosarcoma, cells were treated without or with berberine (Sigma-Aldrich), respectively. To detect the effects of caspase-1 on osteosarcoma, cells were treated without or with selective caspase-1 inhibitor N-Ac-Tyr-Val-Ala-Asp-CMK (Ac-YVAD-CMK) (Cayman Chemical, Ann Arbor, MI, USA), respectively.

MTT assay of cell proliferation. Cell viability was determined by MTT assay according to the manufacturer's instructions. Briefly, cells ($2x10^4$ cells/well) were seeded in a 96-well plate and treated differently based on the experimental purpose. Cells were washed with phosphate-buffered saline (PBS), and then 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (5 mg/ml) was added to each well. The plate was covered and shaken at room temperature, after which the medium was discarded. Next, dimethyl sulfoxide (DMSO) was added to each well (200 μl), and the solution was vigorously mixed to dissolve the purple tetrazolium crystals. The amount of produced purple formazan is proportional to the percentage of cell viability. The absorbance of each well was measured by automated microplate reader at a test wavelength of 490 nm. All experiments were repeated at least three times.

Western blot analysis. Western blotting was used to detect the expression levels of the proteins of interest. Drugs were diluted and added to cells for 24 h at 37˚C before analysis by western blot. The cells were washed using ice-cold phosphate-buffered saline, and total protein was harvested with radioimmunoprecipitation assay buffer (RIPA) containing 1% protease inhibitor (Sigma-Aldrich). Protein (100 μg) per sample was separated using 12% SDS-PAGE, and then transferred into nitrocellulose membranes. The membrane was blocked with 5% non-fat milk (BD Biosciences, San Jose, CA, USA) and 0.1% Tween-20 in Tris-buffered saline and immunoblotted overnight using appropriate primary antibodies at 4˚C with gentle shaking. After that, fluorochrome labelled secondary antibody (Alexa Fluor 800; LI-COR Biosciences, Lincoln, NE, USA) was used to identify the appropriate primary antibody. Immunoreactivity was detected with the Odyssey fluorescent scanning system (LI-COR Biosciences) and analyzed by Image Studio software. β-actin was used as a loading control.

cDNA synthesis and real-time PCR analysis. Real-time PCR was used to measure caspase-1 and IL-1β mRNA levels. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from tissues and cells. First-strand cDNA was synthesized using a reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCRs were carried out with a SYBR-Green PCR Master Mix kit (Applied Biosystems) and performed on 7500 FAST Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as an internal control. The following primers were used in the study. Caspase-1: forward, 5'-ACACGTCTTGCCCTCATTATCT-3' and reverse, 5'-ATAACCTTGGGCTTGTCTTTCA-3'; IL-1β: forward, 5'-CCCTGCAGCTGGAGAGTGTGG-3' and reverse, 5'-TGTGCTCTGCTTGAGAGGTGCT-3'; GAPDH: forward, 5'-ATCACTGCCACCCAGAAGAC3' and reverse, 5'-TTTCT AGACGGCAGGTCAGG-3'.

TUNEL assay. Cells were seeded on coverslips in 6-well culture plates and grown overnight for adherence, then treated with different drugs, respectively. Apoptosis was measured by an *In Situ* Cell Death Detection kit according to the manufacturer's instructions (Roche Applied Science). In brief, cells were fixed with freshly prepared 4% paraformaldehyde for 60 min. The slides were rinsed with PBS and incubated in 0.1% Triton X-100 permeabilization solution for 2 min. Then slides were incubated with TUNEL reaction mixture for 60 min at 37˚C in a humidified chamber. the rinsed slides were counterstained with DAPI. Cells were counted under a fluorescence microscopy and the green fluorescence staining cells were calculated as positive-staining cells. All experiments were repeated at least three times.

Establishment of a xenograft mouse model. The experimental protocols were approved by the Ethic Committee of Harbin Medical University (Harbin, China). The use of animals followed the National Institutes of Health guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). BALB/c-nu/nu mice, male, 5-6-week old weighing 18-20 g were used. The mice were housed with a regular 12-h light/12-h dark cycle and *ad libitum* access to standard rodent chow diet and were kept in a pathogen-free environment. For *in vivo* tracking, the Saos-2 and MG-63 cells were stably transfected with firefly luciferase. Saos-2 and MG-63 cells ($1x10^7$ cells were suspended in 100 μl serum-free DMEM) were injected subcutaneously into the back of mice. Eight days post-implantation, the mice were randomly divided into three groups (n=6 for each group) and fed by oral gavage with saline, berberine (20 mg/kg/day), or berberine and intraperitoneal caspase-1 inhibitor Ac-YVAD-CHO (0.1 mg/kg/day). Tumor growth was monitored by luciferase activity in Saos-2 and MG-63 cells, and the emitted photons from the target site penetrated through the mammalian tissue and could be externally detected and quantified using a sensitive light imaging system. Mice were euthanized 21 days after treatment and the tumors were isolated for further detection.

Statistical analysis. Data are expressed as mean ± standard error of mean (mean ± SEM) and analyzed with SPSS 13.0
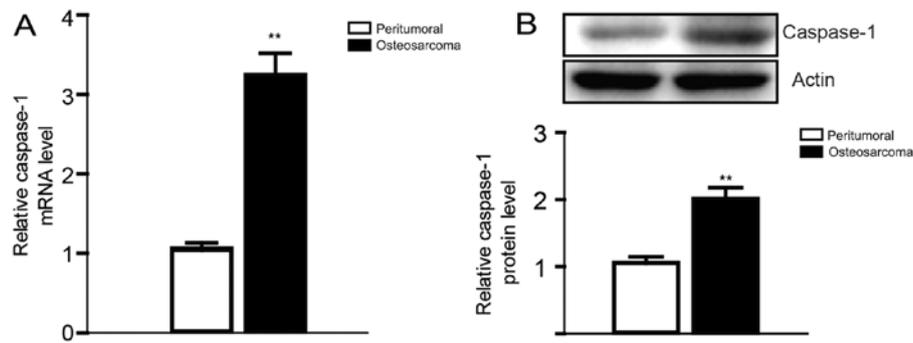
Figure 1. The mRNA and protein expression of caspase-1 were both elevated in six pairs of osteosarcoma tissues compared with peritumoral tissues. (A) Caspase-1 mRNA expression in osteosarcoma tissues was obviously highter than that in peritumorial tissues as determined by real-time PCR. (B) Caspase-1 protein expression in osteosarcoma tissues was obviously highter than that in peritumorial tissues as determined by western blotting. Data are expressed as mean ± SEM. n=3. **P<0.01 when compared with peritumoral control group.
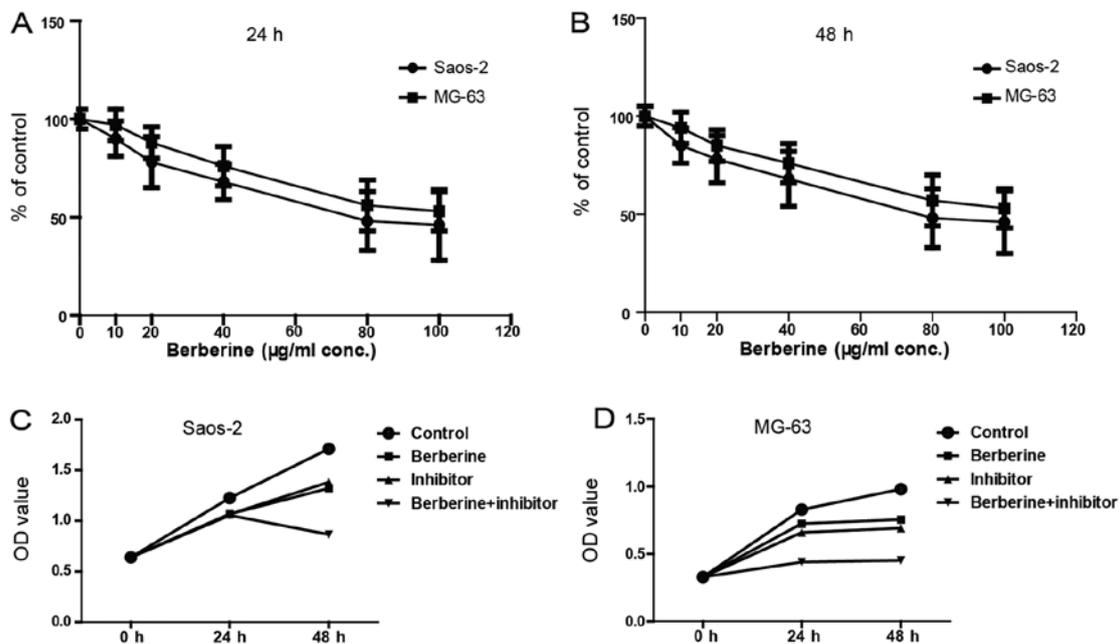
Figure 2. The effects of berberine and caspase-1 inhibitor on the viability of Saos-2 and MG-63 osteosarcoma cells. (A and B) The viability of cells after treatment of different concentration of berberine for 24 and 48 h, respectively. (C and D) The viability of cells after administration of berberine (80 μM) and/or caspase-1 inhibitor, n=6. *P<0.05 when compared with control group.

software. Statistical comparisons between the two groups were performed using the Student's t-test. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). A two-tailed P<0.05 was taken to indicate a statistically significant difference.

Results

The expression of caspase-1 in osteosarcoma tissues. Based on previous studies on the relationship between osteosarcoma and inflammation, and the central role of caspase-1 in the process of inflammation (18-20), we first compared the expression level of caspase-1 in osteosarcoma tissues from six pairs of clinical osteosarcoma cases with peritumoral tissues. Then we found that the expression of caspase-1 was obviously elevated both in the mRNA and the protein level, which is consistent to our expectation (Fig. 1).

Berberine decreases the viability of osteosarcoma cell. Next, we evaluated the effects of berberine on Saos-2 and MG-63 cells by MTT assay. MTT assay results demonstrated that berberine significantly inhibits the growth of Saos-2 and MG-63 cells in a time- and dose-dependent manner. As shown in Fig. 2A and B, the concentration of berberine at 80 μM could inhibit the cell viability to the greatest extent; and the viable cells at 48 h decreased more obviously than 24 h after treatment with 80 μM berberine. Thus, the following administration of berberine were all at 48 h with 80 μM. Fig. 2C and D shows that berberine significantly reduced osteosarcoma cell viability and caspase-1 inhibitor exerts similar effects, which suggest that caspase-1 may be involved in the inhibition of osteosarcoma cell growth caused by berberine.

Berberine induces apoptosis of Saos-2 and MG-63 osteosarcoma cells. To study the effect of berberine administration on
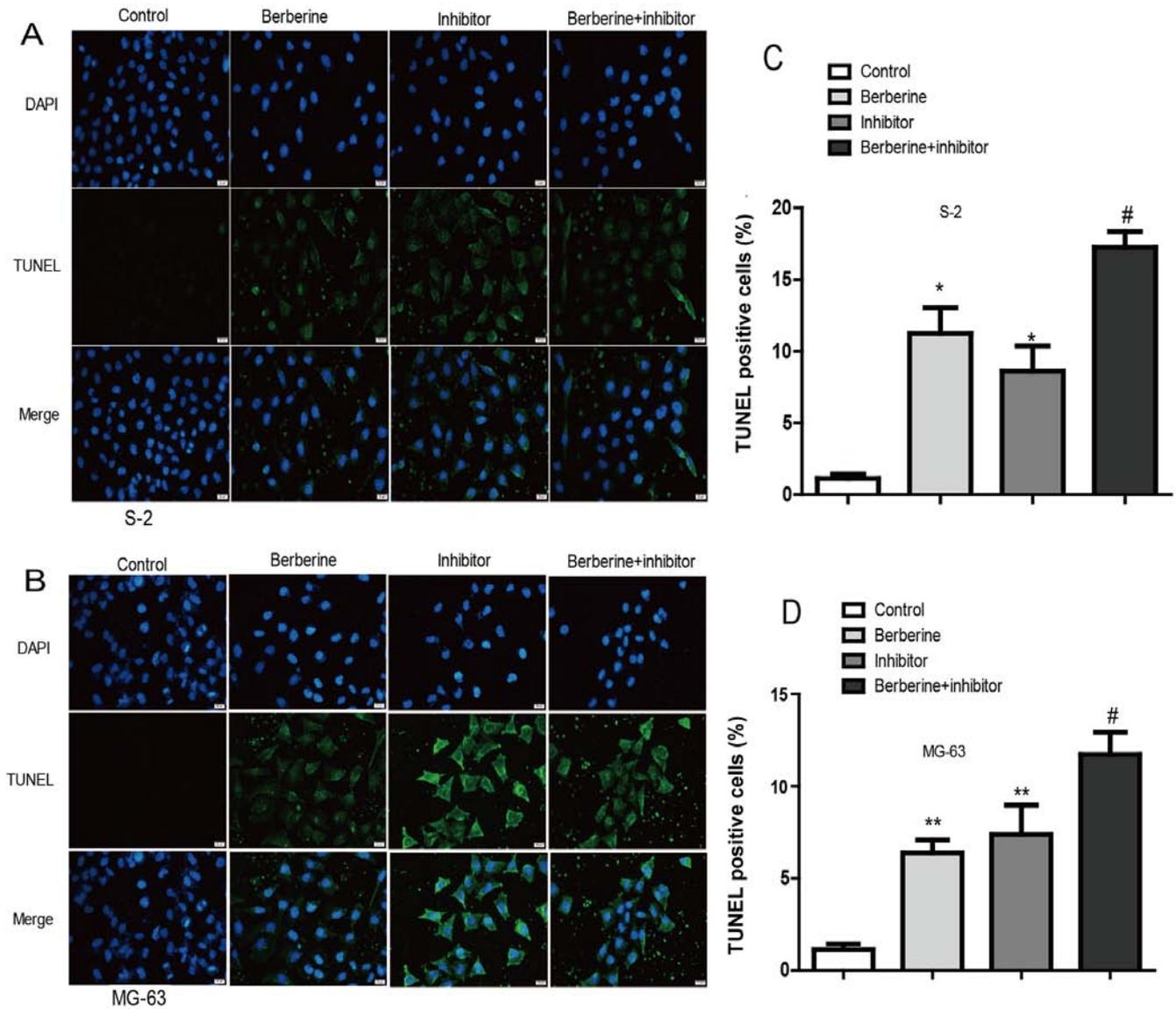
Figure 3. The anti-inflammation effect of berberine induced osteosarcoma apoptosis assessed using TUNEL staining. (A and C) Representative images of TUNEL-positive nuclei in green fluorescent color and total nuclei staining with propidium iodide (PI); (B and D) bar diagram showing the relative proportion of TUNEL-positive cells (n=6). $^*P<0.05$ vs. control, $^{**}P<0.01$ when compared with control group; $^#P<0.05$ vs. berberine group.

osteosarcoma cell apoptosis, TUNEL assay was performed. Cells in the images with green nuclei were considered apoptotic. In Fig. 3, we found few cells with nuclei staining green in the control group. After being exposed to 80 μM berberine for 48 h, ~13.12% of cells showed apoptotic hallmarks. Moreover, caspase-1 inhibitor exert similar effect to berberine. In addition, co-incubation berberine with caspase-1 inhibitor exerted inhibitory effects to the greatest extent. Thus, these results indicated that berberine could induce apoptosis of osteosarcoma cells possibly by caspase-1 involved process.

Berberine exerts anti-osteosarcoma property through reducing caspase-1 and IL-1β expression. Currently, the role of inflammation in cancers has caused widespread concern. It is believed that inflammation could promote the occurrence and development of cancer (21,22). Real-time PCR and western blot assay was performed to explore the molecular

mechanism of berberine on the anti-osteosarcoma property. As we can see from the bar graphs (Figs. 4 and 5), caspase-1 mRNA and protein expression level were both downregulated in Saos-2 and MG-63 osteosarcoma cells after treated with berberine; caspase-1 inhibition extert similar effect to berberine on caspase-1 expression level. At the same time, to gain further insights into the mechanism of anti-osteosarcoma of berberine, we analyzed the expression level of caspase-1 downstream target IL-1β, which plays a decisive role in the formation of tumor inflammatory microenvironment. The results show that the expression of IL-1β was consistent with caspase-1. Furthmore, immunofluorescence staining analysis was used to further confirm the anti-osteosarcoma property of berberine. Accordingly, immunofluorescence results showed that the expression of caspase-1 and IL-1β were both downregulated compared with normal groups. Caspase-1 inhibition exerts similar effect to berberine on osteosarcoma cells (Fig. 6).
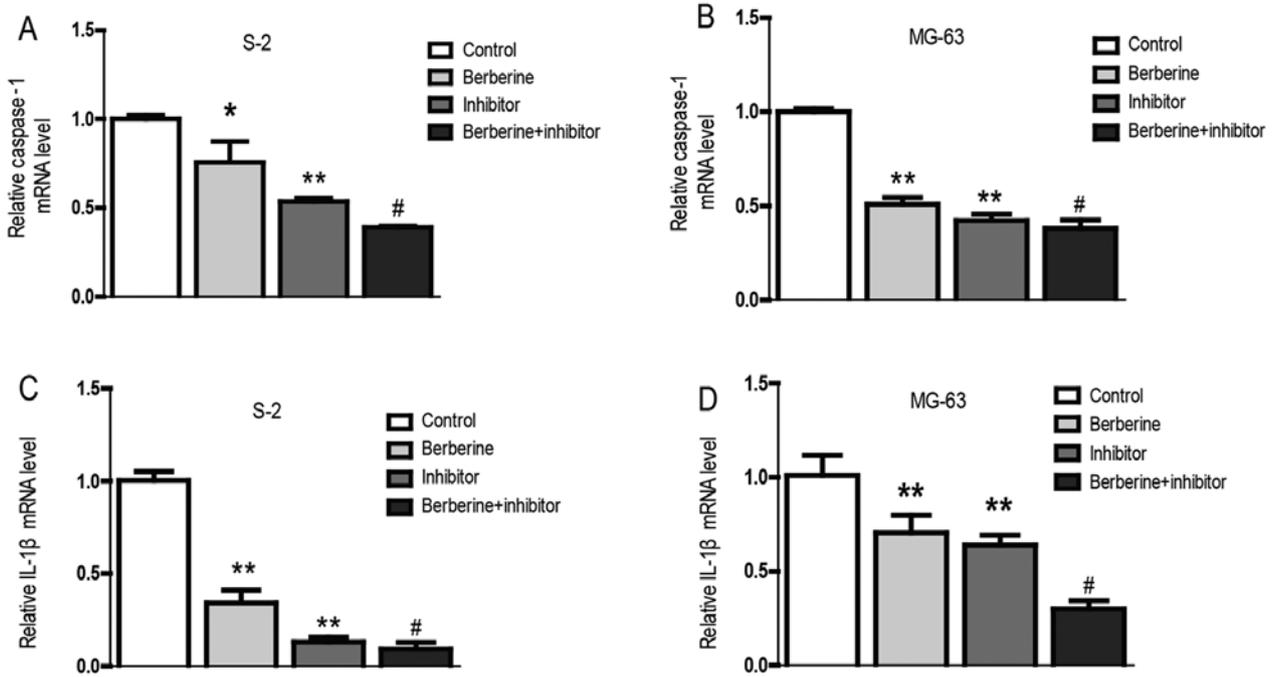
Figure 4. The effects of berberine and/or caspase-1 inhibitor on the mRNA expression level of caspase-1 and IL-1β. After administration of berberine (80 μM) and/or caspase-1 inhibitor for 48 h, the mRNA expression level was detected by real-time PCR. (A) Caspase-1 mRNA expression in the Saos-2 osteosarcoma cells. (B) Caspase-1 mRNA expression in the MG-63 osteosarcoma cells. (C) IL-1β mRNA expression in the Saos-2 osteosarcoma cells. (D) IL-1β mRNA expression in the MG-63 osteosarcoma cells. GAPDH served as an internal control. Data are expressed as mean ± SEM. n=3. *P<0.05 vs. control, **P<0.01 vs. control. #P<0.05 vs. berberine group.
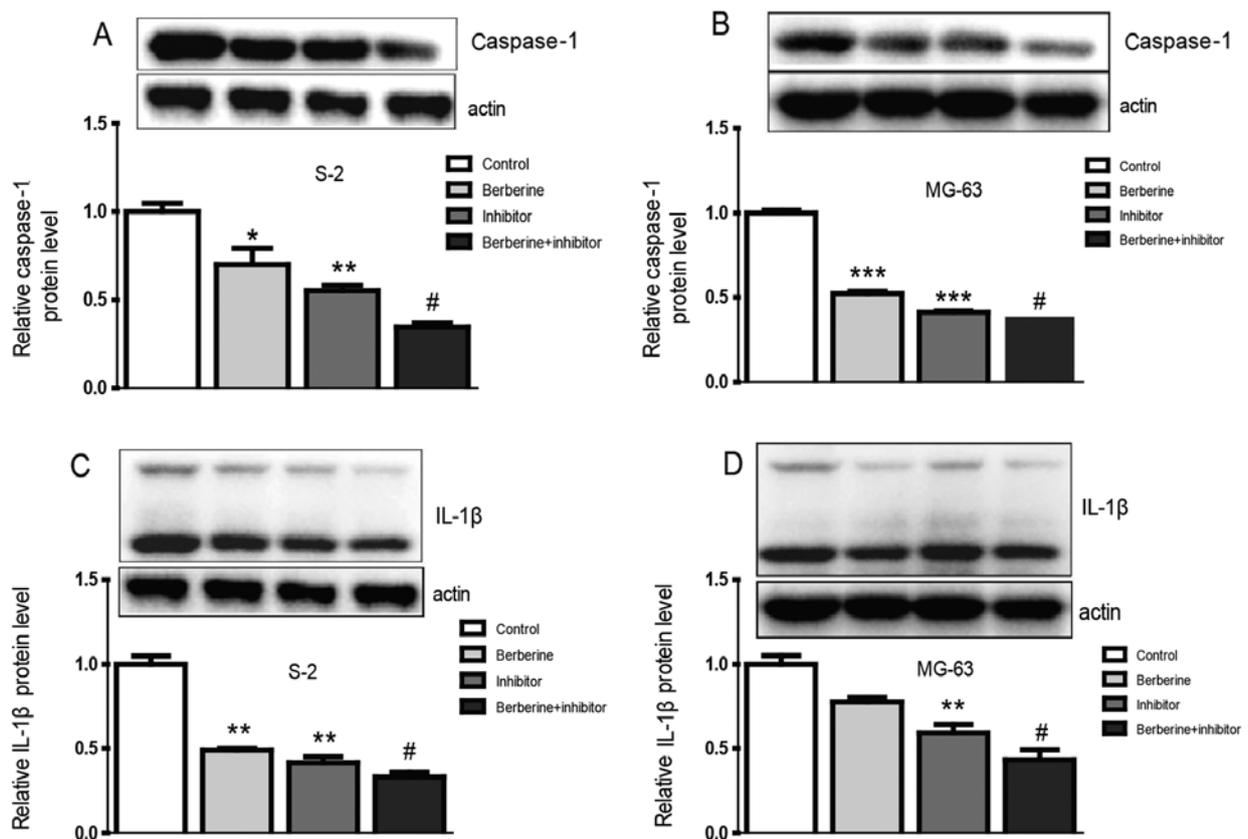
Figure 5. The effects of berberine and/or caspase-1 inhibitor on the protein expression level of caspase-1 and IL-1β. After administration of berberine (80 μM) and/or caspase-1 inhibitor for 48 h, the protein expression level was detected by western blot analysis. (A) Caspase-1 protein expression in the Saos-2 osteosarcoma cells. (B) Caspase-1 protein expression in the MG-63 osteosarcoma cell. (C) IL-1β protein expression in the Saos-2 osteosarcoma cells. (D) IL-1β protein expression in the MG-63 osteosarcoma cells. β-actin served as an internal control. Data are expressed as mean ± SEM. n=3. *P<0.05 vs. control, **P<0.01 vs. control, ***P<0.001 vs. control, #P<0.05 vs. berberine group.
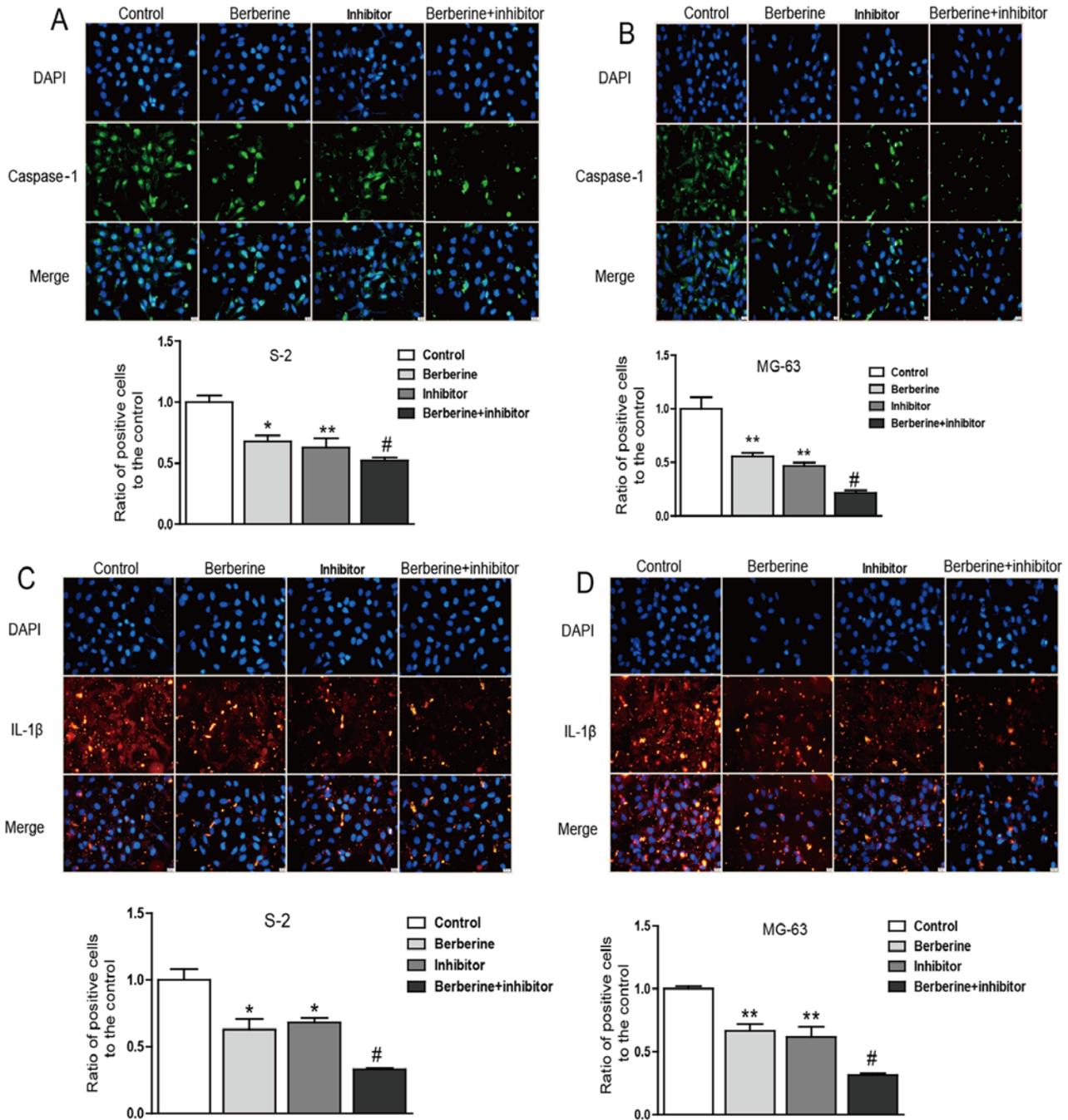
Figure 6. Berberine-induced downregulation of caspase-1 and IL-1β deterimined by immunofluorescence stain. (A-D) Representative images of caspase-1 and IL-1β immunofluorescence in osteosarcoma cells. Data are expressed as mean ± SEM. n=3. *P<0.05 vs. control, **P<0.01 vs. control, $^\#$P<0.05 vs. berberine group.

Berberine inhibits the growth of osteosarcoma tumor in tumor-bearing mice. After eight days of post-implantation of the osteosarcoma cells, the mice were treated differently. Fig. 7 shows that the size of the osteosarcoma shrinks obviously after administration of berberine by oral gavage compared with the control group. Then, the tumor tissues were isolated for further mRNA and protein detection. The results from the tumor tissues were in accordance with the results from Saos-2 and MG-63 osteosarcoma cells (Fig. 8), which illustrate that berberine attenuates the activation of caspase-1/IL-1β signal pathway. In conclusion, these observations demonstrate that berberine could probably relieve the inflammation in tumor

microenvironment and then results in apoptosis of osteosarcoma cells.

Discussion

Chronic inflammation occurring within the microenvironment of tumor lesions is now thought to either drive the first malignant-conferring genetic mutations and/or induce them as a result of the oncogene expression (23). It is believed that inflammation could promote the occurrence and development of tumors (24,25). However, whether relieving the inflammation can attenuate the viability of cancer cells remains

Figure 7. Berberine inhibits the growth of osteosarcoma tumors in tumor-bearing mice. Tumor photographs from each treatment group. The groups in the photograph from the left to the right are as follows: control, berberine, caspase-1 inhibitor and berberine plus caspase-1 inhibitor. The size of the osteosarcoma shrinks obviously after the administration of berberine by oral gavage compared with the control group. The caspase-1 inhibitor exerts similar effect to berberine.

unknown. Caspases are a family of cysteine proteases that play essential roles in inflammation (18). Among the caspase family, caspase-1 is a unique protease because it activates the proinflammatory cytokines IL-1β and IL-18 into their active mature peptides, which play a decisive role in the formation of tumor inflammatory microenvironment and lead to a downstream inflammatory response (26,27).

Over the past decades, interest in the pharmacological effects of natural bioactive compounds with respect to application in cancer treatments and for cancer prevention has greatly increased (28). Natural products represent a rich reservoir of potential small chemical molecules exhibiting various pharmacological effects. Accumulating evidence has demonstrated a dramatic correlation between the natural compounds and cancer prevention and treatment (29-31). Berberine is found in plants from the protoberberine group, such as Berberis, *Berberis vulgaris* and *Berberis aristata*. This kind of plant is traditionally used as a broad-spectrum anti-microbial medicine. During the last few decades, many studies have demonstrated that berberine has anticancer and anti-inflammatory activities. Berberine has drawn extensive attention towards its antineoplastic effects. It seems to suppress the growth of a wide variety of tumor cells, including breast cancer, lung cancer, melanoma and glioma. It has been reported that berberine induces cell circle arrest and apoptosis in human osteosarcoma cells (17,32). The effects of berberine on the osteosarcoma cells have not been systematically investigated and the underlying mechanism is controversial. We tried to explain the mechanism of berberine on osteosarcoma with respect to inflammation and cancer.

In the present study, we investigated the effect of berberine on the osteosarcoma cells, and the mechanism underlying the inhibition of osteosarcoma cell viability after being treated with berberine. Based on our previous study, caspase-1 was significantly elevated in the tissues of osteosarcoma patients (Fig. 1), and the complicated relationship between inflammation and cancer, we a explored whether caspase-1/IL-1β was involved
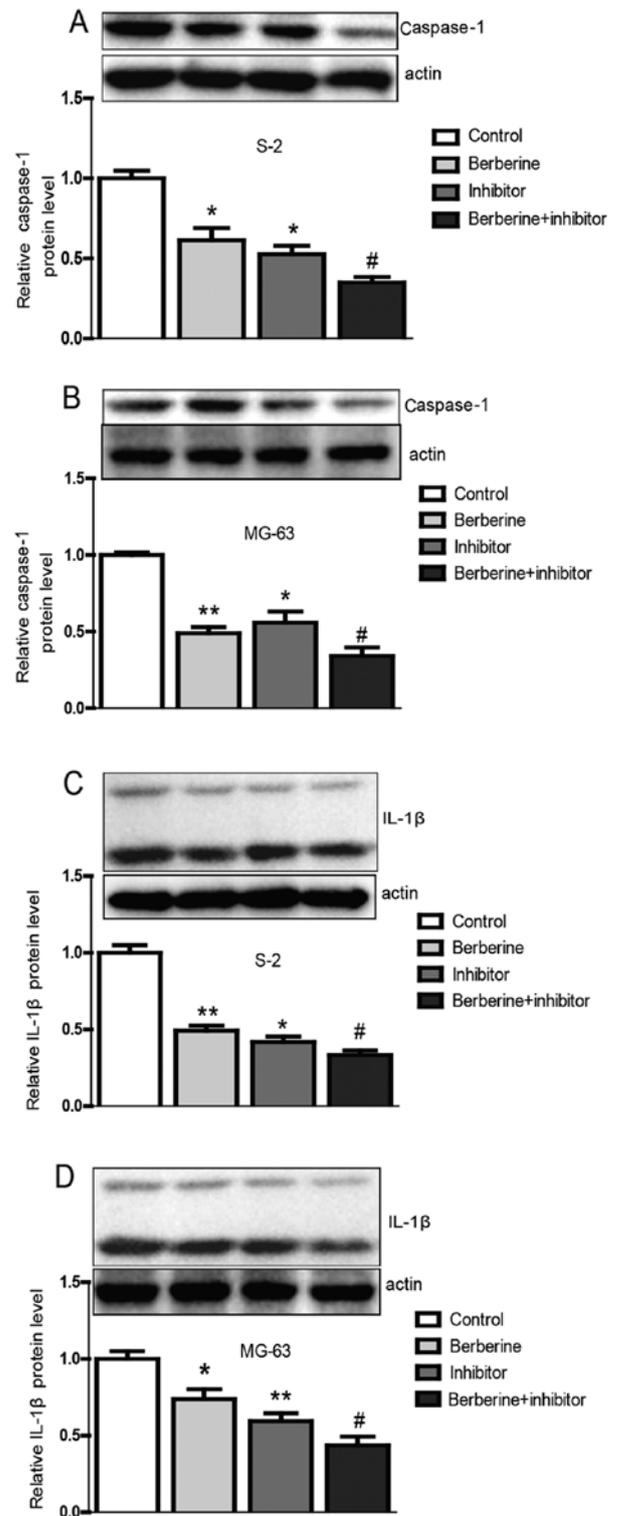
Figure 8. Berberine inhibits the caspase-1/IL-1β singnaling pathway in tumor-bearing mice. (A-D) The expression level of caspase-1 and IL-1β in the osteosarcoma tissues from tumor-bearing mice. The results were in accordance with the previous outcomes from the Saos-2 and MG-63 osteosarcoma cells. Berberine obviouly reduces the mRNA and the protein level of caspase-1 and IL-1β compared with the control group. *P<0.05 vs. control, **P<0.01 vs. control, #P<0.05 vs. berberine group.

in the molecular mechanisms underlying the anticancer property of berberine. Thus, the expression of caspase-1 and IL-1β were assessed. The present results show that caspase-1

and IL-1β in osteosarcoma cells were both downregulated after being treated with berberine *in vivo* and *in vitro*. These results were further confirmed by the adiministration of caspase-1 inhibitor. Caspase-1 inhibition exerts similar effect to berberine. Simultaneously, we established xenograft mouse model to further confirm the anticancer property of berberine. The results confirmed previous findings. Further studies are required to evaluate how berberine alleviates the inflammation in the tumor microenvironment causing apoptosis.

In conclusion, the present study investigated the effect of berberine on osteosarcoma cells, and the relationship between caspase-1/IL-1β signaling pathway and osteosarcoma cell survival implying that inflammation microenvironment could influence the viability of osteosarcoma cells to a great extent. This study suggests that caspase-1/IL-1β could be a new therapeutic target and berberine could be used or as an adjuvant agent in the treatment of osteosarcoma.

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