SPANDIDOS PUBLICATIONS
INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 35: 1729-1733, 2015

Interleukin-32 stimulates osteosarcoma cell invasion and motility via AKT pathway-mediated MMP-13 expression

YANHONG ZHOU[1], ZHAOHUI HU[2], NINGNING LI[2] and RENJIE JIANG[2]

Departments of [1]Clinical Laboratory and [2]Spine Surgery, The People's Hospital of Liuzhou, Liuzhou 545006, P.R. China

Received June 28, 2014; Accepted March 9, 2015

DOI: 10.3892/ijmm.2015.2159

Abstract. As a pro-inflammatory cytokine, interleukin-32 (IL-32) is reported to play an important role in tumor development and progression. However, its effects on the invasion and motility of osteosarcoma cells remain elusive. The aim of the present study was to determine the molecular mechanisms of IL-32 in osteosarcoma cells using RT-PCR and western blot analysis. The results showed that IL-32 stimulation dose-dependently promoted the invasion and motility of osteosarcoma cells. Knockdown of endogenous IL-32 by siRNA inhibited osteosarcoma cell invasion and motility. Moreover, IL-32 induced the activation of AKT in a time-dependent manner. IL-32 stimulation was also capable of increasing the expression and secretion of matrix metalloproteinase (MMP)-13, which is involved in tumor invasion and metastasis. In addition, blockade of AKT activation suppressed IL-32-mediated invasion, motility and MMP-13 upregulation in osteosarcoma cells. Taken together, our results suggest that IL-32 stimulation promotes the invasion and motility of osteosarcoma cells, possibly via the activation of AKT and the upregulation of MMP-13 expression. Thus, IL-32 may serve as a marker for diagnosis, as well as for the treatment of osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone tumor that predominantly affects children and adolescents (1). Although traditional therapies such as chemotherapy, surgery and radiotherapy have been developed, the 5-year survival rates of osteosarcoma patients remain at only 60-70% (2). Invasion and metastasis are responses leading to mortality of osteosarcoma patients. The metastasis of osteosarcoma is mainly hematogenous, and often occurs in the distal organs, such as lungs (3). In many cases, osteosarcomas have already metastasized at the time of diagnosis (4). Therefore, identification of the molecular mechanisms underlying osteosarcoma invasion and metastasis, and improvement of new clinical approaches for the diagnosis and therapy of osteosarcoma is crucial.

Accumulating evidence suggests that the levels of many inflammatory cytokines are increased in the tumor microenvironment, and these inflammatory cytokines play crucial roles in the progression of cancer, including cell growth, invasion and metastasis (5,6). Interleukin-32 (IL-32) is a type of inflammatory cytokine that is mainly produced by T-, natural killer, epithelial cells and monocytes after stimulation by IL-2, IL-18 or IFN-γ (7,8). The *IL-32* gene is located on human chromosome 16p13.3, and has six splice variants, IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε and IL-32ζ (9). Although the receptor for IL-32 remains to be determined, IL-32 has been found to stimulate TNF-α, IL-1β and IL-8 production and thus act as a pro-inflammatory mediator in inflammatory diseases and tumor (7,10). IL-32 expression is increased in malignant esophageal tissues (11), and overexpression of IL-32 is reversely associated with 5-year recurrence-free, disease-specific and overall survival rates in localized clear cell renal cell carcinoma (12). However, the role of IL-32 in osteosarcoma progression remains to be elucidated. In the present study, we focused on the effects of IL-32 on cell invasion and motility, and aimed to determine the molecular mechanisms of IL-32 in osteosarcoma cells.

Materials and methods

Reagents and cell culture. Recombinant IL-32 was purchased from R&D Systems (Minneapolis, MN, USA). AKT selective inhibitor LY294002 was purchased from Calbiochem (San Diego, CA, USA). Antibody of IL-32 was obtained from Abcam (Cambridge, UK). Antibodies of AKT and phosphorylated AKT were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody of β-actin was obtained from Sigma Aldrich (St. Louis, MO, USA). The MG-63 human osteosarcoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in 5% CO_2 at 37˚C in a humidified incubator.

Invasion assay. Cell invasion ability was assessed using an invasion assay. Briefly, a 24-well Transwell plate was

Correspondence to: Dr Zhaohui Hu, Department of Spine Surgery, The People's Hospital of Liuzhou, 8 Wen Chang Road, Liuzhou 545006, P.R. China
E-mail: zhaohuihulz@163.com

Key words: interleukin-32, invasion, motility, AKT, matrix metalloproteinase-13, osteosarcoma

purchased from Costar (Corning, NY, USA) and the upper chambers were coated with Matrigel prior to use. The cells were adjusted to a concentration of $5x10^5$/ml and then treated with different concentrations of IL-32 (0, 50, 100 and 200 ng/ml). Subsequently, 200 μl of cell suspension was added into the upper chambers, and 500 μl DMEM supplemented with 20% FBS was added into the lower chambers. After 18 h, the cells that invaded through the Matrigel-coated filters were fixed and stained with crystal violet. The invaded cells were counted under a microscope at a magnification of x200.

Wound-healing assay. Cell motility was assessed by a scratch wound-healing assay. The cells were seeded in a 6-well plate, cultured until confluent and then treated with or without IL-32 (100 ng/ml). The cell layer was wounded by a sterile tip and the spread of wound closure was observed after 18 h under a microscope at a magnification of x100.

RNA interference (RNAi) assay. Small-interfering RNA (siRNA) was designed and obtained from Shanghai GeneChem (Shanghai, China). Using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), MG-63 cells were transfected with an IL-32 siRNA (5'-GCUCACUCCUCUACUU GAA-3') or a scramble control siRNA (5'-UGGUUUACAUGUUUUC UGA-3'). The cells were then incubated for 48 h and the knock-down efficiency was assessed by western blot analysis.

Western blot analysis. Cell lysates were extracted by RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Applygen Technologies, Inc., Beijing, China), and then the BCA method was used to measure the concentration of total protein. An equal amount of protein was separated by SDS-PAGE gels and then transferred onto a PVDF membrane. The membrane was then blocked for 1 h in TBST containing 5% BSA, and subsequently immunoblotted with primary antibody overnight at 4˚C. After washing with TBST, the membrane was incubated for 1 h with secondary antibody. Finally, the bands were visualized via chemiluminescence using an ECL Detection kit (Applygen Technologies).

RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen), as per the manufacturer's instructions. Reverse transcription was performed to obtain cDNA using the RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada). Subsequently, 2 μg of cDNA was amplified with the primers of matrix metalloproteinase (MMP)-13 (F, 5'-ACTGAGAGGCTCCGAGAAATG-3' and R, 5'-GAACC CCGCATCTTGGCTT-3'); and β-actin (F, 5'-ATAGCACA GCCTGGATAGCAACGTAC-3' and R, 5'-CACCTTC TACAATGAGCTGCGTGTG-3') using a SYBR-Green PCR kit (Applied Biosystems, Carlsbad, CA, USA). RT-PCR analysis was performed using the following cycle parameters: 10 min at 95˚C, and then 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. β-actin was used as internal control and the relative expression of MMP-13 was determined by the $2^{-\Delta\Delta Ct}$ method.

ELISA. The cells were incubated with or without IL-32 (100 ng/ml) for 18 h. The cell supernatant was collected and stored at -80˚C until the ELISA was performed. The MMP-13 protein level in the cell supernatant was assessed by the MMP-13 ELISA kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions.

Statistical analysis. The experiments were performed at least three times. Data were presented as mean ± standard error of the mean. The data were analyzed using SPSS software (version 18.0). Comparisons between any two groups were assessed by the Student's t-test and comparisons among multiple groups were assessed by one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant result.

Results

IL-32 promotes the invasion and motility of osteosarcoma cells. In order to investigate the role of IL-32 in the invasion of osteosarcoma cells, MG-63 cells were stimulated with different concentrations of IL-32 (0, 50, 100 and 200 ng/ml) and then an invasion assay was performed. The results showed that IL-32 stimulation promoted the invasion of MG-63 cells in a dose-dependent manner (Fig. 1A). Furthermore, the effect of IL-32 on cell motility was determined by the wound-healing assay. Notaby, the results showed that IL-32 stimulation led to a significant increase in MG-63 cell motility (Fig. 1B). These results indicated that IL-32 stimulation enhances the invasion and motility of osteosarcoma cells.

Knockdown of IL-32 suppressed the invasion and motility of osteosarcoma cells. To examine whether endogenous IL-32 can affect the invasion and motility of osteosarcoma cells, we silenced the expression of IL-32 in MG-63 cells by siRNA. Western blot analysis revealed that the endogenous expression of IL-32 was efficiently repressed by siRNA IL-32 (Fig. 2A). Resutls of the invasion and wound-healing assays showed that knockdown of IL-32 markedly inhibited the invasion and motility abilities of MG-63 cells, supporting the hypothesis that IL-32 is important in osteosarcoma cell invasion and motility (Fig. 2B and C).

IL-32 stimulation induces the activation of AKT. Multiple signaling pathways have been observed to be activated by IL-32 (13). In the present study, we studied whether IL-32 stimulation affected the activation of AKT in osteosarcoma cells. The results showed that IL-32 (100 ng/ml) induced the activation of AKT in a time-dependent manner, with peak activation occurring at 30 min (Fig. 3), indicating that IL-32 induces the activation of AKT in osteosarcoma cells.

IL-32 increases the expression and secretion of MMP-13. Since the MMPs are essential for the invasion and metastasis of tumor, we assessed whether IL-32 affected MMPs expression in osteosarcoma cells. MG-63 cells were stimulated with or without IL-32 (100 ng/ml) for 12 or 18 h, and the mRNA levels of MMP-2, MMP-9 and MMP-13 were detected by RT-PCR. The results showed that the mRNA expression of MMP-13 in MG-63 cells was markedly increased after IL-32 stimulation (Fig. 4A). Thus, we further examined the effect of IL-32 on MMP-13 secretion in MG-63 cells. ELISA showed that IL-32 stimulation resulted in increased protein secretion of MMP-13 in MG-63 cells (Fig. 4B). These results suggested

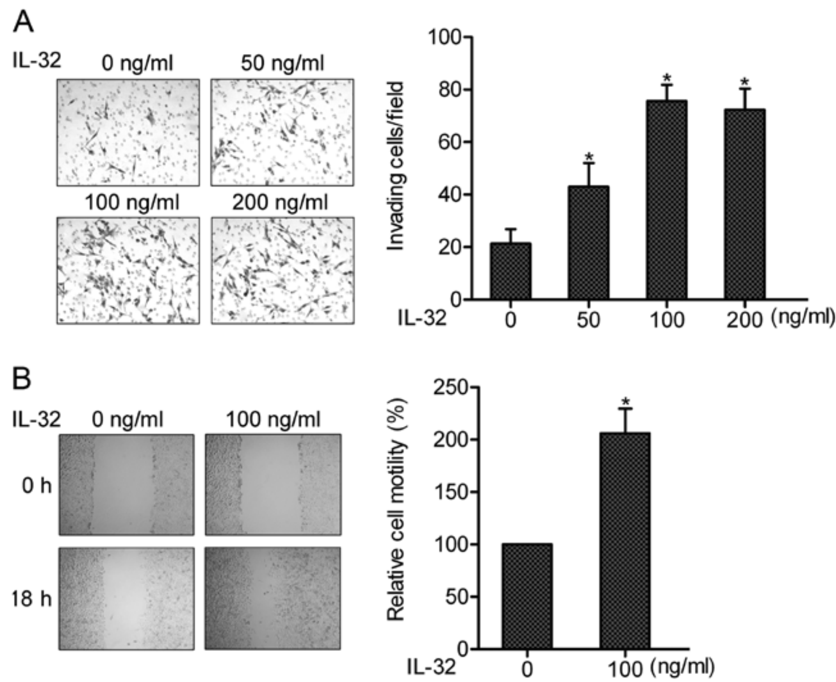

Figure 1. IL-32 stimulation resulted in an increase in osteosarcoma cell invasion and motility. (A) Effect of IL-32 stimulation (0, 50, 100 and 200 ng/ml) on cell invasion was detected by an invasion assay. (B) Effect of IL-32 stimulation (100 ng/ml) on cell motility was detected by a wound-healing assay. *P<0.05.

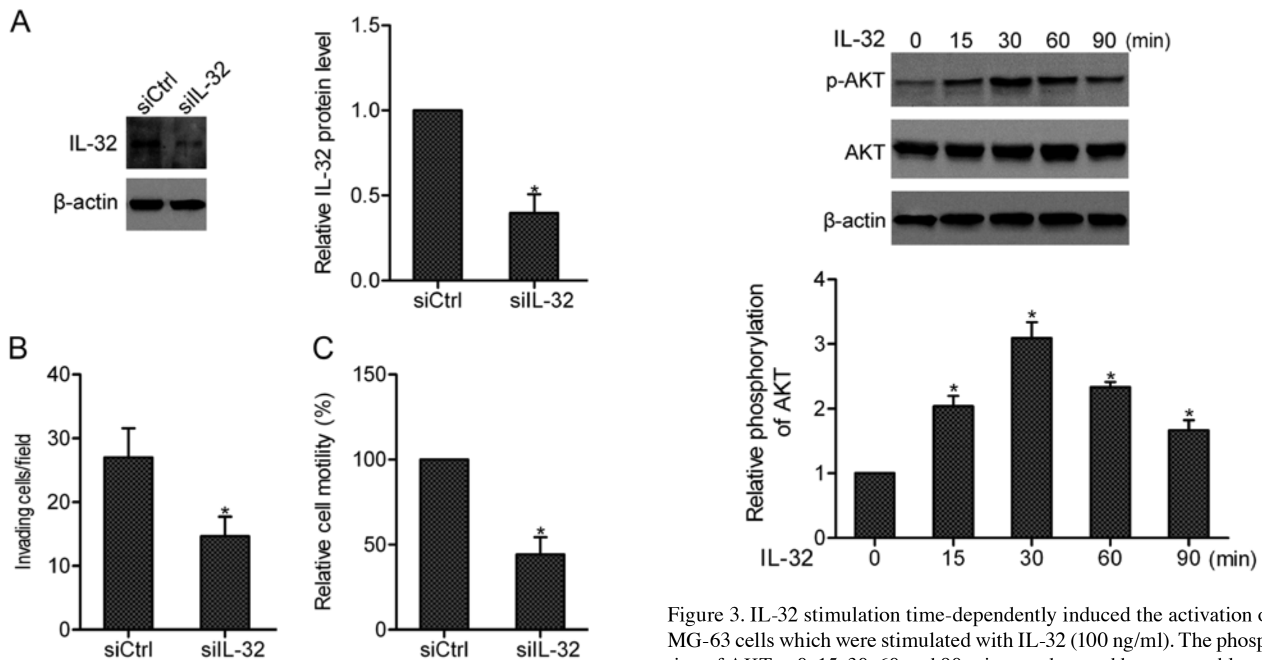

Figure 2. Knockdown of IL-32 inhibited osteosarcoma cell invasion and motility. (A) Western blot analysis showed that IL-32 expression in MG-63 cells was efficiently silenced by IL-32 siRNA. (B) Knockdown of IL-32 suppressed the invasion of MG-63 cells. (C) Knockdown of IL-32 suppressed the motility of MG-63 cells. *P<0.05.

Figure 3. IL-32 stimulation time-dependently induced the activation of AKT. MG-63 cells which were stimulated with IL-32 (100 ng/ml). The phosphorylation of AKT at 0, 15, 30, 60 and 90 min was detected by western blot analysis. *P<0.05.

that IL-32 stimulates the expression and secretion of MMP-13 in osteosarcoma cells.

AKT pathway is involved in IL-32-enhanced cell invasion and motility. To detect the role of AKT pathway in IL-32-mediated invasion and motility, LY294002, a selective AKT inhibitor, was added to MG-63 cells prior to IL-32 stimulation. The results showed that IL-32 stimulated the invasion and motility in the DMSO-treated group. However, after inhibition of AKT by LY294002, the invasion and motility abilities of MG-63 cells were markedly suppressed, suggesting the involvement of AKT pathway in IL-32-enhanced invasion and motility in osteosarcoma cells (Fig. 5).

IL-32 regulates MMP-13 expression and secretion via the AKT pathway. We verified the effect of AKT activation on

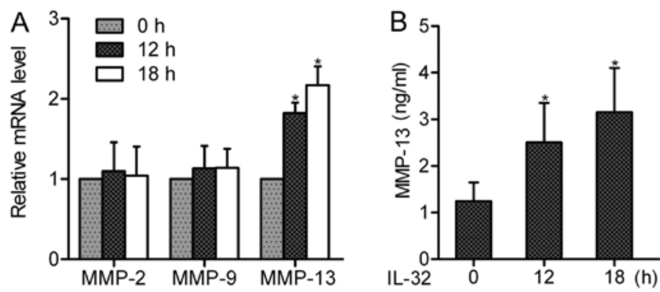

Figure 4. IL-32 upregulated the expression and secretion of MMP-13. (A) The mRNA levels of MMP-2, MMP-9 and MMP-13 were detected by RT-PCR in MG-63 cells stimulated with IL-32 (100 ng/ml). (B) MG-63 cells were treated with IL-32 (100 ng/ml) for 12 or 18 h, and then the protein levels of MMP-13 in the cell supernatant was determined by ELISA assay. *P<0.05.

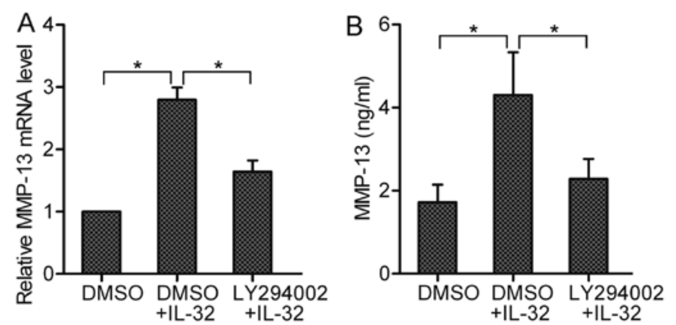

Figure 6. IL-32 upregulated MMP-13 expression and secretion through AKT activation. MG-63 cells were treated with LY294002 (AKT inhibitor, 10 μM) for 30 min prior to IL-32 (100 ng/ml) stimulation. After 18 h, (A) RT-PCR and (B) ELISA assay were performed to detect the effect of AKT blockade on the IL-32-mediated MMP-13 expression and secretion in osteosarcoma cells. *P<0.05.

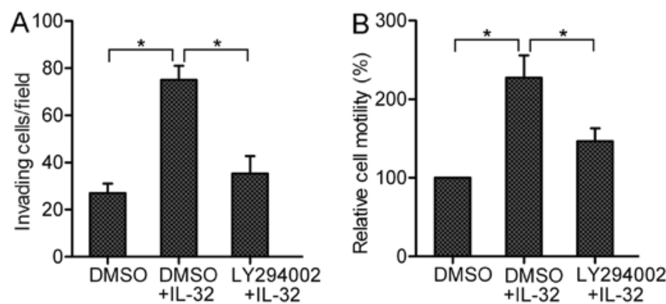

Figure 5. IL-32 promoted osteosarcoma cell invasion and motility via activation of the AKT pathway. MG-63 cells were treated with LY294002 (AKT inhibitor, 10 μM) for 30 min prior to IL-32 (100 ng/ml) stimulation. (A) Invasion and (B) wound healing assays were performed to determine the role of AKT pathway in IL-32-mediated cell invasion and motility. *P<0.05.

the IL-32-regulated MMP-13 production. RT-PCR and the ELISA showed that when AKT activation was blocked by LY294002 prior to IL-32 stimulation, the mRNA expression and protein secretion of MMP-13 induced by IL-32 stimulation were significantly inhibited (Fig. 6), suggesting that IL-32 upregulates MMP-13 expression and secretion dependent on AKT activation.

Discussion

As a member of the inflammatory cytokines, IL-32 has been found to be invovled in the progression of cancer. Previous findings have shown that IL-32 exerts antitumor activity by inhibiting cell growth and inducing cell apoptosis in various types of cancer, such as colon cancer and hepatocellular carcinoma (14,15). However, IL-32 is able to promote the tumorigenesis of colon cancer cells (16), and stimulate the angiogenesis of endothelial cells (17). IL-32 has been shown to be associated with the invasion and metastasis of gastric and lung cancer (18,19). Moreover, experimental data have demonstrated that IL-32 stimulates the migration of breast cancer cells (20), and increases the invasion and metastasis of gastric and lung cancer cells (21,22), indicating that IL-32 is a crucial mediator for tumor invasion and metastasis. IL-32 acts as a potent modulator of osteoclastogenesis (23); however, little is known concerning the effect of IL-32 on osteosarcoma. In the present study, we found that IL-32 stimulation dose-dependently increased the invasion and motility of osteosarcoma cells, and knockdown of endogenous IL-32 by siRNA significantly inhibited osteosarcoma cell invasion and motility. These results strongly support the hypothesis that IL-32 contributes to the invasion and motility of osteosarcoma cells.

Although the receptor for IL-32 is not well established, studies have proven that IL-32 stimulation leads to the activation of multiple signaling pathways, including ERK1/2, p38 and nuclear factor (NF)-κB pathways (24,25). It is reported that IL-32 treatment induces a massive activation of AKT in osteoclast (23). Previous findings have shown that IL-32 stimulates the activation of AKT in gastric cancer cells (21). Similarly, we found that IL-32 stimulation time-dependently induced the activation of AKT. The AKT pathway is known to be essential for tumor invasion and metastasis (26). In the present study, inhibition of the AKT pathway markedly attenuated IL-32-enhanced osteosarcoma cell invasion and motility, indicating that IL-32 may promote the invasion and motility of osteosarcoma cells by activating the AKT pathway.

MMP-13 is an important member of the MMP family, and plays a pivotal role in tumor cell invasion and metastasis processes via the degradation of the extracellular matrix (ECM) (27). In the present study, we showed that IL-32 upregulated the expression and secretion of MMP-13 in osteosarcoma cells. In addition, we found that the AKT pathway was required for IL-32-mediated MMP-13 upregulation. Previous studies have reported the involvement of MMP-13 in the regulation of osteosarcoma progression (28-30). Therefore, it is possible that AKT pathway-mediated MMP-13 upregulation participates in IL-32-promoted osteosarcoma cell invasion and motility.

In summary, the present study has demonstrated that IL-32 is capable of promoting the invasion and motility in osteosarcoma cells. The activation of AKT and subsequent upregulation of MMP-13 production contributes to the biological functions of IL-32. Thus, IL-32 acts as a potential therapeutic target for osteosarcoma.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (no. 81260274).

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