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Original Article

IL-1β promotes cervical cancer through activating NF-κB/CCL-2

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Abstract: Cervical cancer is a malignancy with high morbidity and mortality among women. Interleukin (IL)-1β, chemokine (C-C motif) ligand 2 (CCL-2), and activation of NF-κB have been proven to be closely related to the progression of various tumors. However, their role in cervical cancer remains unclear. Cell proliferation, migration, and invasion were detected using MTT, wound healing, and transwell assays. Western blotting and qRT-PCR were used to measure expression of target genes. IL-1β greatly promoted the release of CCL-2 from HeLa cells. Activation of NF-κB and phosphorylated NF-κB (p65) nuclear translocation were accelerated by IL-1β. TPCA-1, a blocker of NF-κB, significantly inhibited the release of CCL-2 from HeLa cells. TPCA-1 markedly reversed the promotional effect of IL-1β on viability of HeLa cells. IL-1β increased the cell migration, proliferation, and invasion of HeLa cells through targeting the NF-κB/CCL-2 pathway. IL-1β/NF-κB/CCL-2 might be a promising treatment target for cervical cancer treatment and prevention.

Keywords: IL-1β, NF-κB, CCL-2, HeLa cells, cervical cancer

Introduction

Cervical cancer is one of four major malignant tumors among women. It is reported that cervical cancer cells can affect immune response, and cause immune escape [1, 2], One possible mechanism is that tumor cells limit the host's effective immune response by producing immune regulatory factors, including IL-10, TGF-β, and CCL2, which are involved in the production of tumor microenvironment. Previous study indicated that that CCL2 might be a marker of solid tumor prognosis [3]. In addition, the high expression of CCL2 in several types of tumorsincluding prostate, breast, lung, stomach, colorectal, esophagus and uterus has been observed [4]. CCL2 could be expressed in many types of cervical cancer cell lines, but it was only produced in 30% of normal cervical epithelial cells [5]. The specific mechanism has not been reported.

NF-κB is a transcription factor, and it can promote inflammation, and regulate apoptosis and tumor immune response [6]. The activation of NF-κB is related to the differentiation and invasion of tumor cells. It was reported that the expression of NF-κB (p65) in grade II, III, and IV cervical cancer tissue was significantly higher than that of normal cervical tissues, suggesting that the expression of NF-κB (p65) might be related to tumor grade [7, 8].

A previous study has reported that IL-1β could promote breast cancer through affecting CCL-2 [9, 10]. In addition, IL-1β has been proven to be involved in the promotion of hepatocellular carcinoma cell migration caused by fatty acid oxidation [11]. IL-1β can accelerate pancreatic ductal adenocarcinoma through activating the hedgehog pathway [12]. However, whether IL-1β can promote cervical cancer through inducing the release of CCL2 from HeLa and activating NF-κB remains unclear.

In the present study, we first investigated the effect of IL-1β on cell migration, proliferation, and invasion of cervical cancer cells. The induction release of CCL-2 and activation of NF-κB by IL-1β were measured. Finally, the involvement of NF-κB in the influence of IL-1β on cervical cancer cells viability was observed. This study

might provide new thoughts for the prevention and treatment of cervical cancer.

Materials and methods

Cell culture and treatment

HeLa cells were inoculated into a 50 mL culture flask with 10% fetal bovine serum, and cultured at 37°C, 5% CO_2 for 48 h. Cells were digested using 2.5 g/L trypsin EDTA, then cells were inoculated into the 96-well plate for experiment. DMEM medium without serum was used to dilute IL-1β with a final concentration of 10 ng/ml. 100 μL IL-1β (10 ng/mL) was added to each well, and HeLa cells were stimulated for different time courses (0 h, 1 h, 6 h, 9 h, 12 h, 24 h). For 0 h group, HeLa cells were treated with serum-free DMEM for 24 h as blank control. Cell culture supernatants were stored at -80°C condition. In the concentration gradient experiment, IL-1β was prepared into different concentration reagents (0.1, 1, 3, 10 ng/mL) using serum-free medium. HeLa cells were starved for 24 hours before the treatment of IL-1β. HeLa cells treated with serum-free medium were used as blank control.

TPCA-1 treatment

HeLa cells were incubated with different concentrations of TPCA-1 (0, 0.1, 0.3, 1, 3, and 10 μmol/L) for 30 min. Then, IL-1β (10 nmol/L) was added to stimulate for 9 h. The supernatant of HeLa cells was collected. All the above experiments were repeated three times. CCL2 was detected using ELISA kit (Nanjing Jiancheng, Nanjing, China). CCL2 level in supernatant of cells was detected according to instructions.

RNA extraction and qRT-PCR

The total RNA was extracted using Trizol method. The first strand of cDNA was synthesized with Easyscript first strand cDNA synthesis Supermix kit. The upstream primer sequence of CCL2 gene was: 5'-AGCAAGTCCAAAGAAGC-3', and the downstream primer sequence was: 3'-TCACCCAAGTCCCTAAGGTAG-5'. The RT-PCR system was SYBR Premix Ex Taq (10 μL), primers (0.4 μL), cDNA template (2 μL), and ddH_2O (7.2 μL). PCR amplification procedure was as follows: pre-denaturation at 95°C for 15 min; denaturation at 95°C for 10 s, annealing at 60°C for 20 s, annealing at 72°C for 32 s, 40 cycles in total. The ratio of CCL2 to 18s mRNA level was calculated by 7300 system SDS software attached to ABI7300. The mRNA level of the experimental group was determined by comparing the value of $\Delta\Delta C_t$.

Western blot

Cells were lysed with lysis buffer (Nanjing Jiangcheng, China). Same amount of protein was loaded for 10% SDS-PAGE. Gels were transferred to a PVDF membrane (Nanjing Jiancheng, China) electrophoretically. 5% nonfat milk was applied for blocking. After 2 h, membrane was cultivated using primary antibodies at 4°C overnight. After washing twice, secondary antibodies were used for incubation for 2 h. After washing twice with TBST buffer, Image J software was used to analyze protein bands. The antibodies used in this study were listed as follows: Rabbit monoclonal to p-NF-κB (ab76302, 1:1000; Abcam, UK), Rabbit monoclonal to NF-κB (ab32536, 1:1500; Abcam, UK), Goat anti-rabbit IgG (ab205718, 1:2000; Abcam, UK).

Cell migration

The cells were prepared with DMEM and seeded into a 6-well plate. When cell confluence reached to 80%, a 100 μL pipette tip was used to make a wound in the middle of plate. The cell number in the wound line was counted at 0 and 48 h.

Cell invasion

Digested cells were seeded on the top chamber of the Transwell plate, 200 μl cell suspension was added to the upper chamber, and DMEM medium containing 10% FBS was added to the lower chamber. After 24 h culture at 37°C 5% CO_2, cells were fixed using 4% polymethanol, and stained using Giemsa. The cells that crossed chamber were photographed and counted.

Cell proliferation

MTT assay (Nanjing Jiangcheng, China) was used to detect cell proliferation. Cells (6×10^4) were plated into a 96-well plate and cultivated for 24 h. After treatment with different

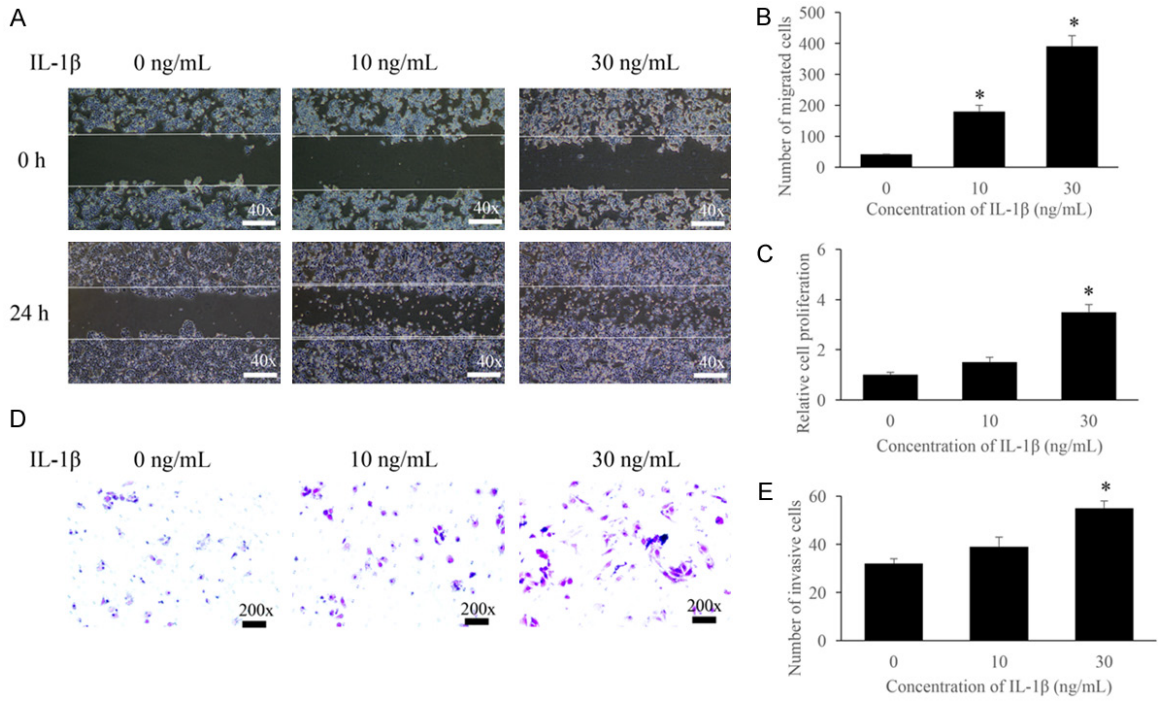

Figure 1. IL-1β promoted the cell migration, proliferation, and invasion of HeLa cells. A: The influence of IL-1β on HeLa cell migration was measured (Scale bar = 500 μm, magnification: 40 ×). B: The influence of IL-1β on HeLa cell migration was quantified. C: The influence of IL-1β on HeLa cell proliferation was measured. D: The influence of IL-1β on HeLa cell invasion was measured (Scale bar = 200 μm, magnification: 200 ×). E: The influence of IL-1β on HeLa cell invasion was quantified. *P<0.05 compared with group 0, ng/mL IL-1β.

administrations, cells were cultured for 24 h. Cell proliferation was detected using according to instruction. After incubation with MTT reagent for 2 h, optical density at 490 nm was measured.

Statistical analysis

The data were expressed as mean ± standard deviation and analyzed by SPSS11.0 software. Single factor analysis of variance was used to compare the data between groups, and non-parametric analysis was used when the variance between groups was uneven.

Results

IL-1β markedly promoted the cell migration, proliferation, and invasion of HeLa cells

Three different concentrations of IL-1β were applied to measure the influence of IL-1β on the cell migration, proliferation, and invasion of HeLa cells. We found that 10 and 30 ng/mL IL-1β significantly increased the migration ability of HeLa cells (**Figure 1A**, **1B**). Meanwhile, high concentration of IL-1β markedly promoted cell proliferation of HeLa cells (**Figure 1C**). In addition, the invasion ability of cells was significantly increased after treatment with 30 ng/mL IL-1β (**Figure 1D**, **1E**). These results indicated that IL-1β might play a key role regulating the viability of HeLa cells.

Influence of IL-1β on CCL-2 release from HeLa cells

In order to detect the ability of IL-1β inducing HeLa to release CCL-2, HeLa cells were stimulated with different concentrations of IL-1β (0, 0.1, 1, 3, 10 ng/ml). IL-1β could induce the release of CCL-2, and there was a significant concentration correlation. Significant release of CCL-2 from HeLa cells were observed after treatment with 1 ng/mL or higher concentration IL-1β. When the concentration of IL-1β reached 10 ng/mL, the content of CCL-2 was 2.6 times of basic value (**Figure 2A**). Different treatment time courses of IL-1β were performed to verify whether IL-1β (10 ng/mL) can cause sustained release of CCL-2. We found that secretion of CCL-2 increased with the time of

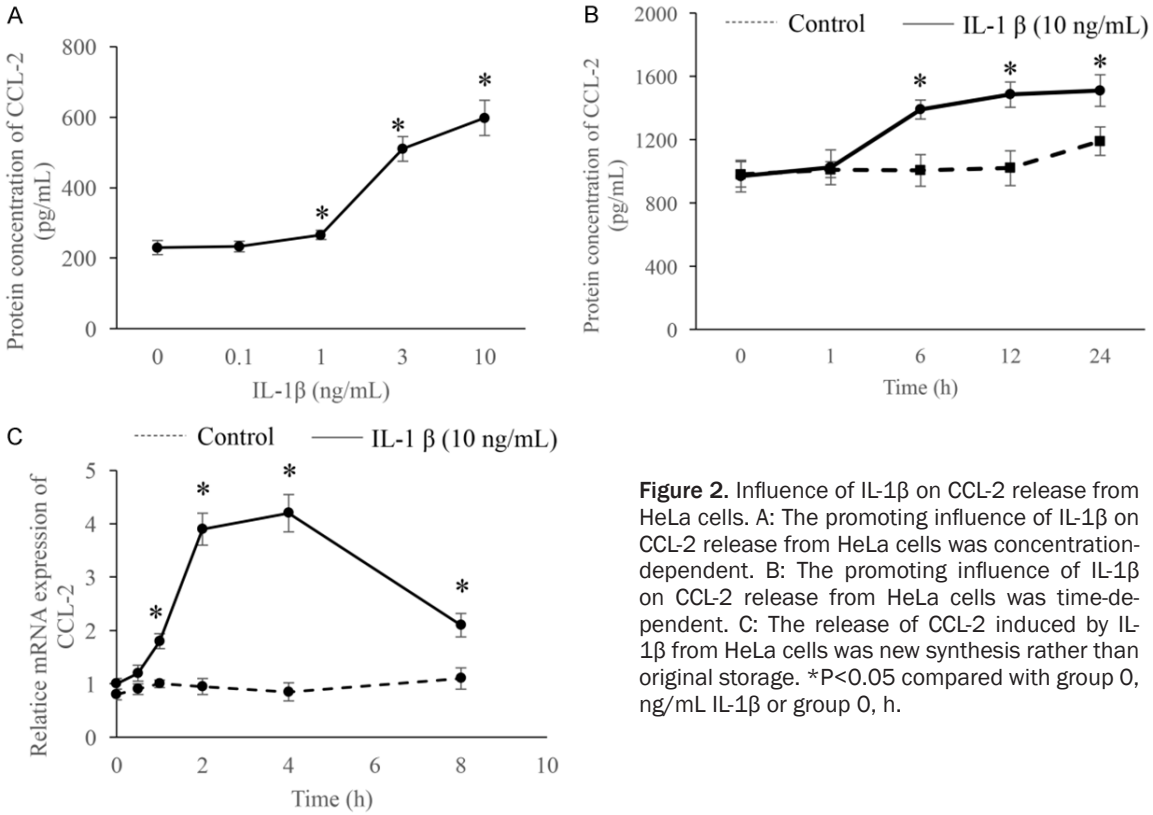

Figure 2. Influence of IL-1β on CCL-2 release from HeLa cells. A: The promoting influence of IL-1β on CCL-2 release from HeLa cells was concentration-dependent. B: The promoting influence of IL-1β on CCL-2 release from HeLa cells was time-dependent. C: The release of CCL-2 induced by IL-1β from HeLa cells was new synthesis rather than original storage. *P<0.05 compared with group 0, ng/mL IL-1β or group 0, h.

IL-1β stimulation, and it was time-dependent (**Figure 2B**).

In order to observe whether the increase of CCL-2 was due to the release of CCL-2 protein in HeLa cells or the synthesis of CCL-2, IL-1β (10 ng/mL) was used to stimulate HeLa cells with different time courses (0, 0.5, 1, 2, 4, 8 h), and the mRNA expression of CCL-2 in cells was measured. Compared with the control group at 0 h, the mRNA level of CCL-2 began to increase at 1 h, and reached a peak at 4 h (**Figure 2C**). The results indicated that IL-1β could up-regulate CCL-2 mRNA in HeLa cells suggesting that the release of CCL-2 induced by IL-1β from HeLa cells was new synthesis rather than original storage.

Influence of IL-1β on NF-κB activation and phosphorylated NF-κB (p65) nuclear translocation

In order to investigate whether NF-κB can be activated by IL-1β, p-NF-κB (p65) was set as a marker of NF-κB activation. IL-1β (10 ng/mL) was used to stimulate HeLa cells with different time courses (0, 5, 10, 30, 60, 120 min). Then the protein expression of p-NF-κB (p65) and NF-κB (p65) total protein were measured. After 5 minutes, p-NF-κB p65 protein expression was up-regulated significantly, and reached a peak at 10 minutes. Then the level of p-NF-κB (p65) decreased gradually, but was still higher than control. However, the total protein level of NF-κB (p65) was not affected by the stimulation by IL-1β (**Figure 3A**, **3B**). The results suggested that IL-1β could induce the activation of NF-κB protein in HeLa cells.

NF-κB mediated transcription activation is related to its cytoplasmic nuclear translocation. The extracted cytoplasmic and nuclear protein levels were measured. We found that with the increase of IL-1β incubation time, p-NF-κB (p65) in cytoplasm increased at 10 min, reached to peak at 30 min, and decreased gradually (**Figure 3C**, **3D**). Meanwhile, the p-NF-κB (p65) level in nucleus increased after 10 min, and reached a maximum at 1 h (**Figure 3C** and **3E**). The translocation of p-NF-κB (p65) from cytoplasm to nucleus was confirmed.

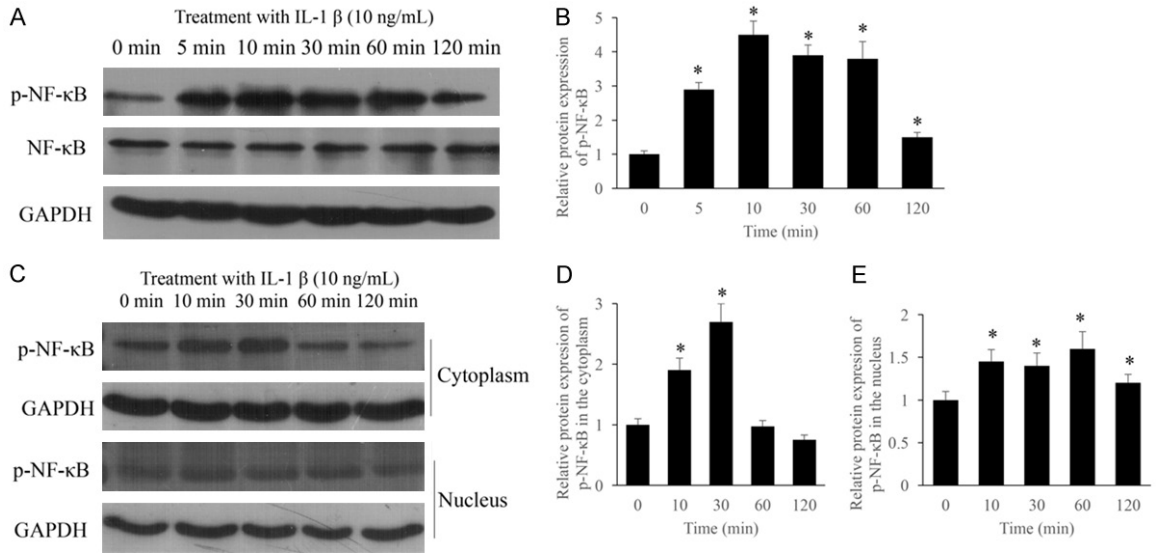

Figure 3. Influence of IL-1β on NF-κB activation and phosphorylated NF-κB (p65) nuclear translocation. A: Influence of IL-1β on NF-κB activation in HeLa cells. B: Quantification of IL-1β Influence on NF-κB activation in HeLa cells. C: Influence of IL-1β on phosphorylated NF-κB (p65) nuclear translocation in HeLa cells. D: Quantification of IL-1β Influence on NF-κB expression in the cytoplasm. E: Quantification of IL-1β Influence on NF-κB expression in the nucleus. *P<0.05 compared with group 0 min.

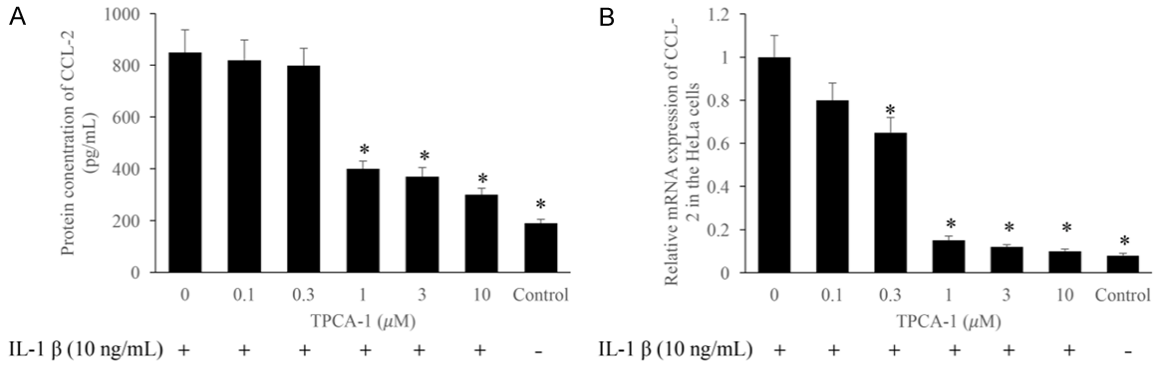

Figure 4. Influence of NF-κB inhibitor on IL-1β stimulated CCL-2 release from HeLa cells. A: Influence of TPCA-1 on IL-1β stimulated CCL-2 release from HeLa cells. B: Influence of TPCA-1 on IL-1β stimulated CCL-2 in HeLa cells. *P<0.05 compared with group 0 μM TPCA-1.

Influence of NF-κB inhibitor on IL-1β stimulated CCL-2 release from HeLa cells

A blocker of NF-κB was used to investigate the effect of IL-1β on CCL-2 expression. We found that TPCA-1 blocked the release of CCL-2 from HeLa induced by IL-1β in a concentration-dependent manner. When TPCA-1 concentration was 1 μM, 55% of the release of HeLa CCL-2 induced by IL-1β was blocked. CCL-2 was decreased by 73% after treatment with 10 μM TPCA-1 (**Figure 4A**). We further investigated the mRNA level of CCL-2 in the HeLa cells, and found that an obvious blocking effect was observed when TPCA-1 concentration was 0.1 μM. When the concentration was 1 μM or higher, around 90% of CCL-2 expression was blocked (**Figure 4B**).

Influence of NF-κB inhibitor on IL-1β stimulated cell migration, proliferation, and invasion of HeLa cells

In order to investigate the role of NF-κB on IL-1β-stimulated cell migration, proliferation, and invasion of HeLa cells, TPCA-1 was used to treat HeLa cells. We found that IL-1β markedly increased the proliferation, migration, and

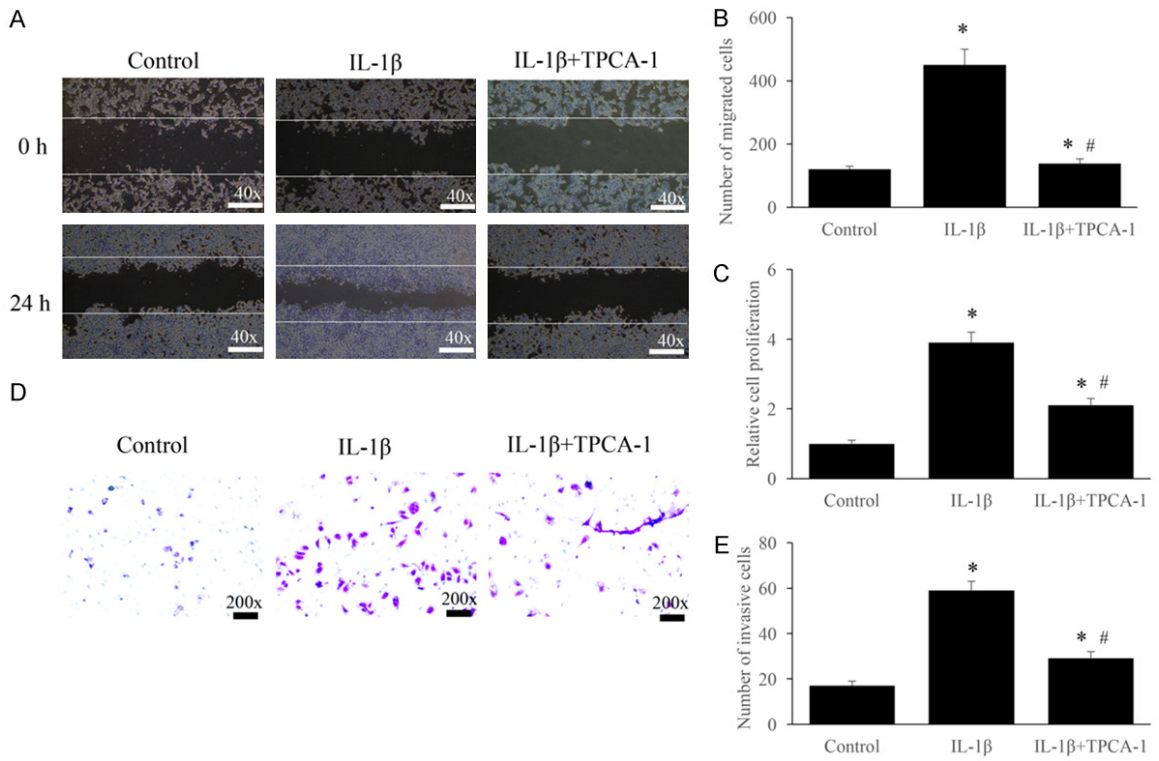

Figure 5. Influence of NF-κB inhibitor on IL-1β-stimulated cell migration, proliferation, and invasion of HeLa cells. A: Influence of TPCA-1 on IL-1β-stimulated migration of HeLa cells (Scale bar = 500 μm, magnification: 40 ×). B: Quantification of TPCA-1 influence on IL-1β-stimulated migration of HeLa cells. C: Influence of TPCA-1 on IL-1β-stimulated proliferation of HeLa cells. D: Influence of TPCA-1 on IL-1β-stimulated invasion of HeLa cells (Scale bar = 200 μm, magnification: 200 ×). E: Quantification of TPCA-1 influence on IL-1β-stimulated invasion of HeLa cells. *P<0.05 compared with group control. #P<0.05 compared with group IL-1β.

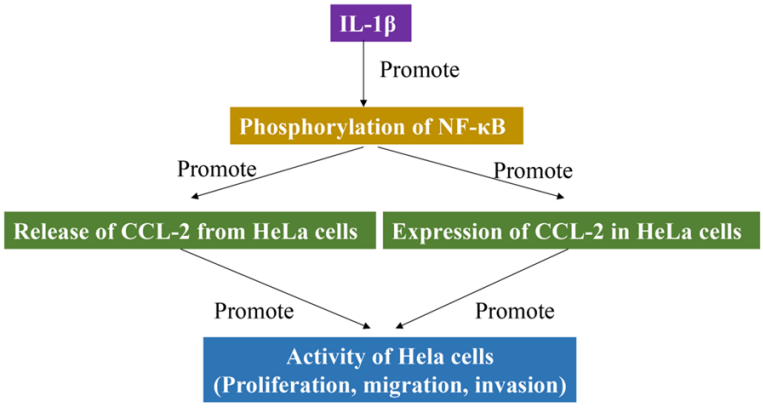

Figure 6. Schematic image indicates the association of IL-1β and NF-κB/CCL-2 pathway.

invasion ability of HeLa cells (**Figure 5A-E**). However, simultaneous administration with IL-1β and TPCA-1, the proliferation, migration, and invasion ability of HeLa cells were markedly suppressed (**Figure 5A-E**). These findings indicated that NF-κB might involve the stimulation process of IL-1β on cell migration, proliferation, and invasion of HeLa cells.

Discussion

IL-1β plays a vital role in inflammation, the immune response, and the pathogenesis of autoimmune diseases. IL-1β can combine with its related receptors, and further activate downstream MAPK, NF-κB, and other related signal pathways. Thereby, the synthesis and expression of downstream chemokines such as gro-2 and CXCL9 is induced [13-15]. However, whether IL-1β can induce the release of CCL-2 and NF-κB activation in HeLa has not been reported.

The transcription factor NF-κB protein in the nucleus of B cells can be induced by lipopolysaccharide (LPS) [16]. It involves DNA binding,

interaction with IκB, and dimerization. Many dimer forms of NF-κB have been detected, but the typical form of NF-κB is a heterodimer of p65/rela and p50 subunits [17, 18]. In HeLa and CaSki cells, the overexpression of NF-κB p65 significantly enhanced the invasion and migration of cervical cancer cells [19, 20]. We observed the upregulation of p-NF-κB (p65) protein expression in HeLa cells after IL-1β stimulation. Our findings indicate that NF-κB mediates the release of HeLa CCL-2 induced by IL-1β.

Based on the findings of this study, we have a preliminary understanding of the regulation of IL-1β on the expression of CCL-2 in HeLa cells and its mechanism, but there are still some problems to be further studied. First of all, IL-1β can regulate CCL-2 expression by activating NF-κB, but this needs to be further confirmed by later experiments and clinical studies. Secondly, with the formation and establishment of inflammatory microenvironment in tumor tissue, the up-regulation of CCL-2 induced by inflammatory factors such as TNFα, IL-1, and IFN-γ may be the main mechanism to promote further secretion of CCL-2 by tumor cells [21-23].

IL-1β can stimulate the synthesis and expression of CCL-2 in HeLa cells. NF-κB (p65) protein was phosphorylated and activated by nuclear translocation after treatment with IL-1β. In addition, synthesis of CCL-2 by HeLa was confirmed. TPCA-1 specifically inhibited NF-κB activation, then the expression of CCL-2 in HeLa cells was significantly decreased. Therefore, IL-1β stimulated the synthesis and release of CCL-2 in HeLa cells by activating NF-κB (**Figure 6**).

It was reported that CCL-2 was believed to be a target gene of NF-κB promoting the migration and invasion of glioblastoma [24]. In the present study, we demonstrated that IL-1β might promote cervical cancer through activating the NF-κB/CCL-2 pathway. This study might provide novel understanding for therapeutic and prevention strategies of cervical cancer.

Disclosure of conflict of interest

None.

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