Bradykinin stimulates IL-6 production and cell invasion in colorectal cancer cells

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Abstract. Bradykinin (BK) has been reported to be involved in the progression of many types of cancer. In the present study, we investigated a possible role of BK in colorectal cancer cell invasion and migration. Invasion and migration assays showed that BK treatment promoted the invasion and migration of colorectal cancer cells. Further experiments showed that BK treatment stimulated ERK1/2 activation and IL-6 production. Two bradykinin receptors, bradykinin B1 receptor (B1R) and bradykinin B2 receptor (B2R), were significantly expressed in all the tested colorectal cancer cells. Repression of B2R, but not B1R, attenuated the BK-mediated invasion and migration, and inhibited ERK1/2 activation and IL-6 production. Moreover, blocking of the ERK pathway decreased the BK-mediated IL-6 production. In addition, IL-6 repression suppressed the effects of BK on colorectal cancer cell invasion and migration. Taken together, the present study demonstrated that BK increases IL-6 production via B2R and the ERK pathway, thereby contributing to the invasion and migration of colorectal cancer cells. Thus, our findings may provide benefits for the treatment of colorectal cancer.

Introduction

As a member of the kinin group, bradykinin (BK) is an active peptide that is generated by the kallikrein-kinin system (KKS) (1). It is reported that BK is involved in the regulation of various cellular processes in cancer cells including tumorigenesis, angiogenesis, cell cycle and proliferation (2). BK exerts its biological functions mainly via the binding of bradykinin receptors, which are pharmacologically characterized as bradykinin B1 receptor (B1R) and bradykinin B2 receptor (B2R) (3). B1R and B2R both belong to the G-protein coupled receptors (4). B1R induces COX-2 production and cell migration in glioma cells (5), whereas B2R promotes VEGF expression and increases angiogenesis in prostate cancer cells (6). Therefore, activation of these two receptors by bradykinin may be essential for the development and progression of cancer.

Colorectal cancer is one of the most common malignancies worldwide. Over 600,000 deaths from colorectal cancer are estimated to occur annually worldwide, thus ranking it as the fourth cause of death among cancer patients (7). Although therapeutic strategies have been well developed, colorectal cancer often progresses, along with cell invasion and metastasis (8). Invasion and metastasis are still the major cause of cancer-related mortality (9). Studies have found that BK can induce MMP-3 expression in human colonic myofibroblasts, which may then contribute to the pathophysiology underlying colitis-associated cancer (10). However, the role of BK in colorectal cancer remains elusive. In this study, we examined the effect of BK treatment on the invasion and migration of colorectal cancer cells. We further characterized the function of B2R in BK-mediated invasion and migration, and determined the possible underlying mechanisms.

Materials and methods

Reagents. The antibodies against B1R (rabbit monoclonal antibody), B2R (mouse monoclonal antibody), β-actin (mouse monoclonal antibody) and ERK1/2 (rabbit polyclonal antibody) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibody against phospho-ERK1/2 (rabbit polyclonal antibody) was obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). BK and PD98059 were purchased from Sigma (St. Louis, MO, USA). IL-6 ELISA kit was purchased from Boster (Wuhan, China).

Cell culture. Human colorectal cancer cell lines, T84, Caco-2, HT-29, HCT116 and SW480, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco-BRL) at 37˚C in a humidified CO_2 incubator.

Invasion assay. A 24-well Transwell plate, containing PET membranes with 8-μm pores, was purchased from

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Costar (San Diego, CA, USA). The upper chambers of the plate were coated with thin layers of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Cells ($1.0x10^5$ cells in 0.2 ml DMEM/well) were pretreated with different dosages of bradykinin, and then were added to the upper chambers. The chemoattractant (20% FBS) was placed into the lower chambers. After incubation in a CO_2 incubator for 16 h, the cells that invaded through the membrane were fixed and stained with eosin. The invaded cells were observed under a microscope (magnification, x100), and the number of the invaded cells was counted from five random fields. All experiments were conducted in triplicate, and the data are presented as a percentage of the invaded cells in the control.

Migration assay. The procedure for the migration assay was similar to the invasion assay described above, except that the upper chambers were coated without Matrigel. Briefly, cells were adjusted to the concentration of $5.0x10^5$ cells/ml. Two hundred microliters of the cell suspensions was placed in the upper chambers, while 20% FBS was placed into the lower chambers. Sixteen hours later, the migrated cells were stained with eosin. The number of migrated cells was counted from five random fields, and the data are presented as a percentage of the migrated cells in the control.

Western blot analysis. Cells were lysed in 200 μl lysis buffer, which contained 50 mM Tris-HCl (pH 7.5), 1% NP-40, 150 mM NaCl, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM Na_3VO_4 and 1 mM NaF. The protein concentrations were determined by BCA assay. Equal amounts of cell lysate (50 μg) were resolved by 10% SDS-PAGE and transferred to a PVDF membrane (Pall Corp., Alcobendas, Spain). After blocking with 5% non-fat milk (for detection of B1R, B2R, β-actin and ERK1/2) or 5% BSA (for detection of p-ERK1/2) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4˚C. Then the membranes were washed with TBST for three times and further incubated with the secondary antibodies for 1 h at room temperature. The bands were detected with an enhanced chemiluminescence kit (Applygen Technologies Inc., Beijing, China), according to the manufacturer's instructions. The density of the bands was quantified with Quantity One software.

siRNA transfection. siRNAs were designed and chemically synthesized by Genepharma (Shanghai, China). The sequences of the siRNAs are listed as follows: control siRNA, 5'-UGG UUUACAUGUUUUCUGA-3'; B1R siRNA, 5'-GCUCCC UGAAUCCAGUAAU-3'; B2R siRNA, 5'-GGCAGAGGA AGAUAUUUCU-3'; IL-6 siRNA, 5'-CCCGGACCAUAU UUAUUAU-3'. Cells were transfected with siRNAs using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) when the cell confluency reached 30-40%. The knockdown efficiency was determined by western blotting or real-time PCR.

ELISA assay. The level of IL-6 in the culture supernatant was observed by ELISA assay, according to the manufacturer's instructions. Briefly, cells were treated with or without BK for 16 h, and then 50 μl of the cell supernatant was added into the ELISA plate wells to detect the level of IL-6. The absorbance

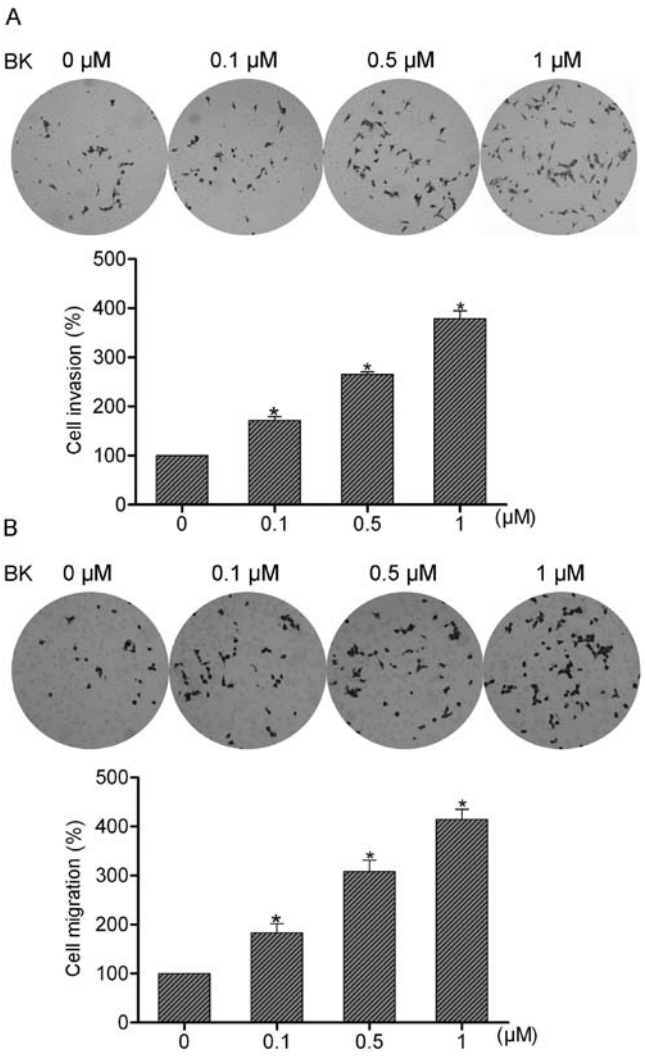

Figure 1. SW480 cells were pretreated with different concentrations of BK (0.1-1 μM), and then subjected to invasion and migration assays. (A) Effect of BK treatment on the invasion of SW480 cells. (B) Effect of BK treatment on the migration of SW480 cells. *P<0.05.

was measured at 450 nm in a microplate reader. The concentration of IL-6 was examined by comparing the absorbance values against the standard curve. The level of IL-6 in each sample was quantified to the total protein.

Real-time PCR. Total-RNA was isolated from the SW480 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentrations of RNA were measured with NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The reverse transcription PCR reaction was carried out with 2 μg of RNA and M-MLV reverse transcriptase (Tiangen, Beijing, China) to obtain cDNA. Then the real-time PCR reaction was performed with cDNA and primers. Oligonucleotide primers are listed as follows: IL-6 sense, 5'-TGCGTCCGTAGTTTCCTTCT-3' and antisense, 5'-GCCTCAGACATCTCCAGTCC-3'; β-actin sense, 5'-AGAAGGATTCCTATGTGGGCG-3' and antisense, 5'-CATGTCGTCCCAGTTGGTGAC-3'. The reaction was performed for 5 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec, and 60˚C for 1 min.

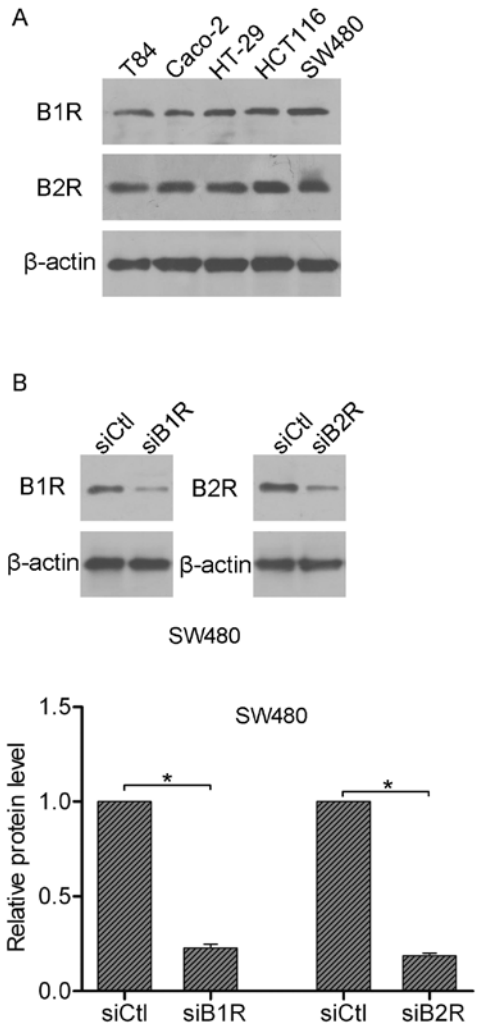

Figure 2. Protein levels of B1R and B2R in colorectal cancer cells. (A) Western blot analysis of B1R and B2R protein expression in T84, Caco-2, HT-29, HCT116 and SW480 cells. (B) Suppression of B1R and B2R expression by siRNA. Cells were transfected with B1R siRNA (siB1R), B2R siRNA (siB2R) or control siRNA (siCtl), respectively. The protein expression of B1R and B2R in SW480 cells was determined by western blotting. *P<0.05.

Statistical analysis. All experiments were repeated three to four times, and the data are presented as the mean ± SEM. The data were analyzed by the Student's t-test between two groups or one-way ANOVA between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

BK enhances the invasion and migration of colorectal cancer cells. To examine the role of BK in colorectal cancer cell invasion and migration, SW480 cells were treated with 0.1, 0.5 or 1 μM BK, respectively. Then the treated or untreated cells were subjected to invasion and migration assays. The invaded or migrated cells were observed and quantified 16 h later. The data showed that BK treatment resulted in a concentration-dependent increase in the invasive and migratory abilities of the SW480 cells (Fig. 1A and B), indicating that BK may be an important modulator of colorectal cancer cell invasion and migration.

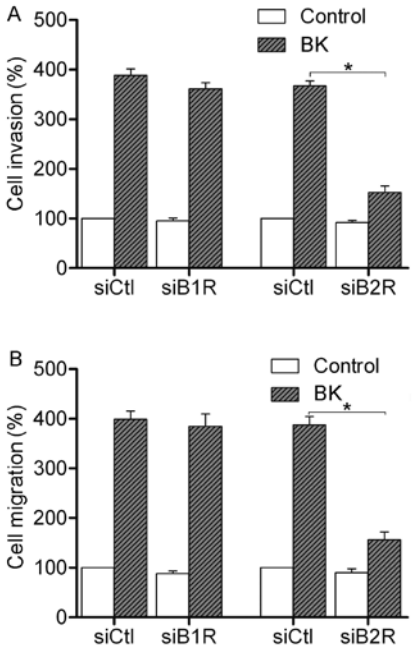

Figure 3. Effects of B1R and B2R on the invasion and migration of SW480 cells. After suppression of B1R or B2R by siRNAs, cells were treated with or without 1 μM BK. Subsequently, (A) invasion and (B) migration assays were carried out using Transwell plates. *P<0.05.

Expression of bradykinin receptors in colorectal cancer cells and suppression of bradykinin receptors by siRNA. It is known that BK acts via bradykinin receptors, which are further characterized as B1R and B2R (3). Here, we detected the expression of B1R and B2R in colorectal cancer cells. As shown in Fig. 2A, all tested cell lines expressed significant amounts of B1R and B2R protein. Next, a B1R siRNA was generated to silence the expression of B1R, while a B2R siRNA was generated to silence the expression of B2R. As observed by western blot analysis, both B1R siRNA and B2R siRNA achieved prominent knockdown efficiency in the SW480 cells (Fig. 2B).

B2R suppression leads to inhibition of BK-mediated invasion and migration in colorectal cancer cells. We further tested the effect of RNAi-mediated B1R and B2R suppression on the invasion and migration of colorectal cancer cells. Invasion and migration assays were performed after BK treatment. As shown in Fig. 3, 1 μM BK treatment promoted the invasion and migration in the control siRNA cells. Suppression of B1R had little effect on the BK-mediated invasion and migration. However, suppression of B2R resulted in a reduction in BK-mediated invasion and migration in SW480 cells. Together, these data suggest that it is B2R that participates in the BK-mediated invasion and migration of colorectal cancer cells.

B2R is required for BK-mediated ERK1/2 activation. Previous studies have shown that BK can induce multiple intracellular signaling pathways of cancer cells including the ERK pathway (11). However, the relationship between BK and the ERK pathway in colorectal cancer cells is, to our knowledge, not yet clear. We, therefore, stimulated SW480 cells with

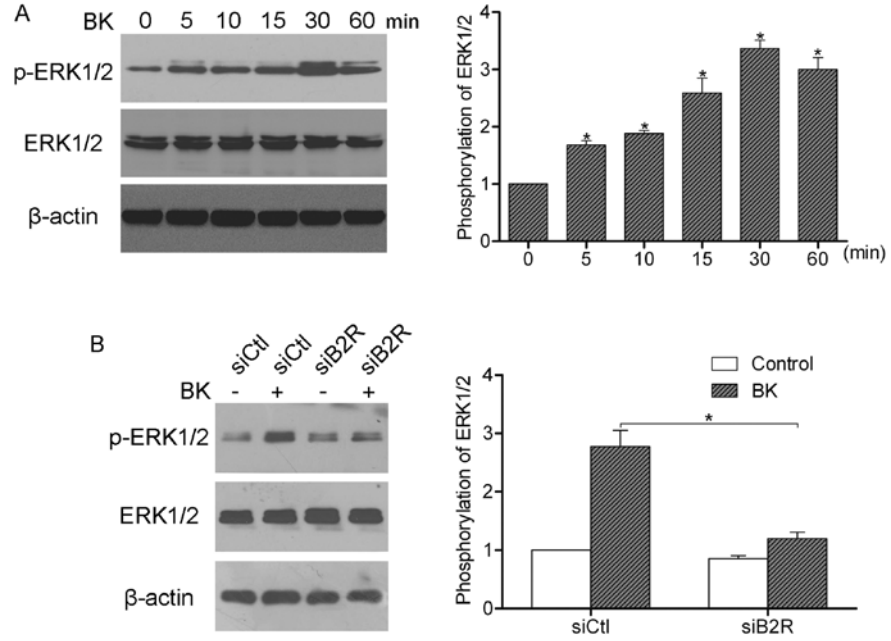

Figure 4. BK induces the activation of ERK1/2 via B2R. (A) SW480 cells were treated with 1 μM BK, and the phosphorylation of ERK1/2 was assessed within 1 h by western blot analysis. (B) After transfection with B2R siRNA (siB2R) or control siRNA (siCtl), SW480 cells were incubated with 1 μM BK or without for 30 min. Western blotting was performed to examine the phosphorylation of ERK1/2. *P<0.05.

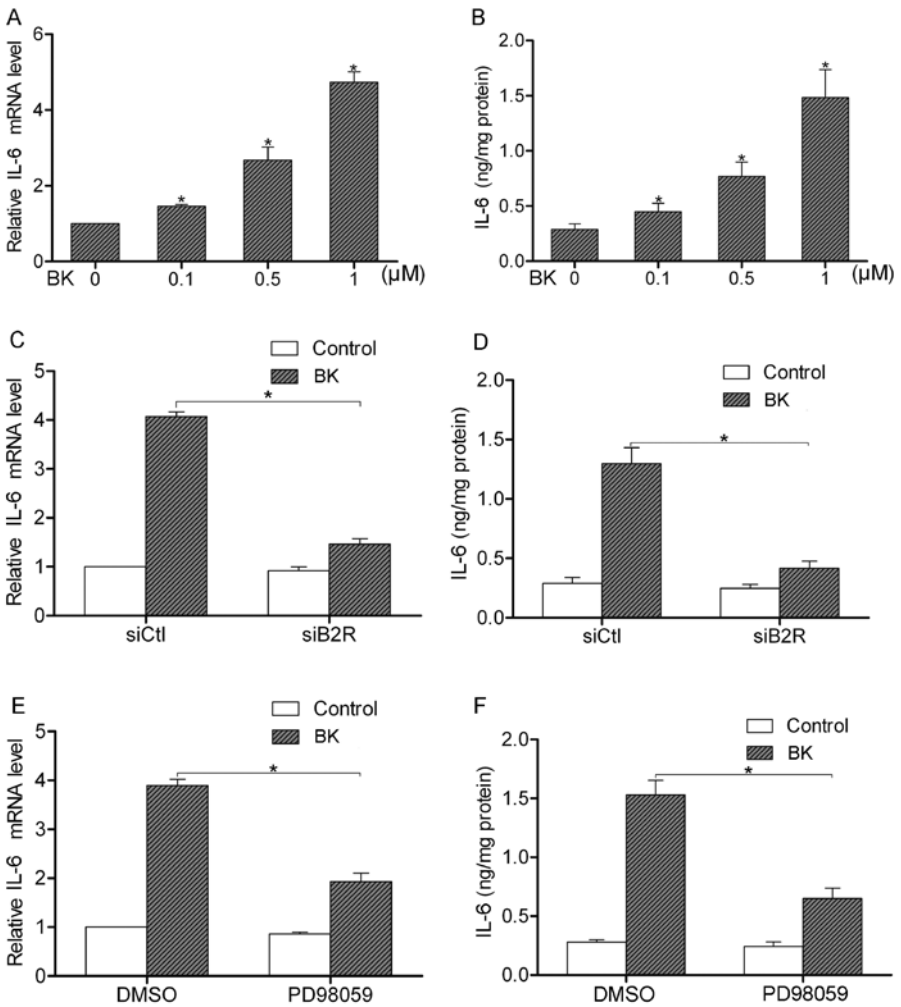

Figure 5. The B2R-ERK pathway contributes to BK-induced IL-6 production. (A and B) Cells were incubated with different concentrations of BK for 16 h and the mRNA level of IL-6 was detected by real-time PCR (A) and the secretion of IL-6 was examined by ELISA assay (B). (C and D) Effects of B2R on the expression and secretion of IL-6. (E and F) Effects of ERK1/2 on the expression and secretion of IL-6. *P<0.05.

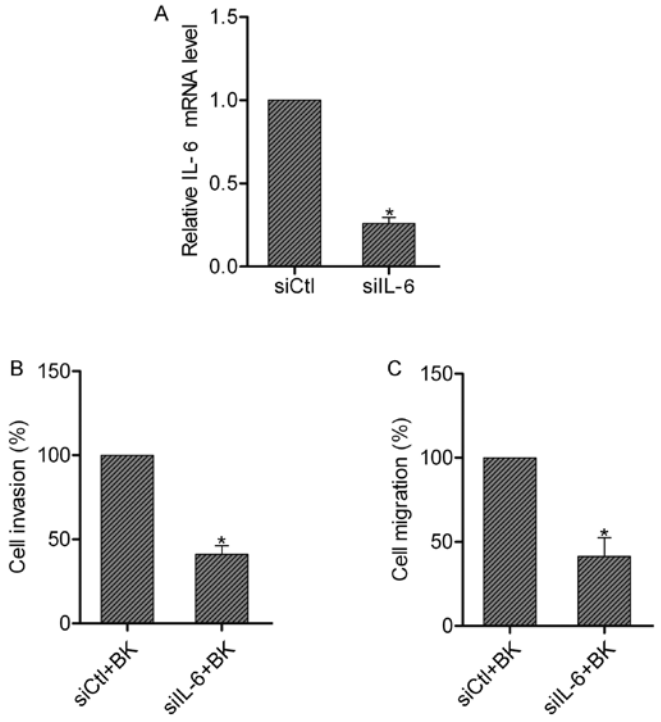

Figure 6. IL-6 is involved in the BK-enhanced invasion and migration in SW480 cells. (A) IL-6 mRNA expression was determined by real-time PCR 48 h after transfection with IL-6 siRNA (siIL-6) or control siRNA (siCtl). (B and C) After transfection with IL-6 siRNA or control siRNA, cells were incubated with 1 μM BK for 16 h. Subsequently, (B) invasion and (C) migration were determined by Transwell assay. *P<0.05.

1 μM BK for 60 min, and then examined the phosphorylation of ERK1/2 by western blot analysis. The results showed that BK treatment induced the activation of ERK1/2, and the peak activation was observed after 30 min (Fig. 4A). Furthermore, we silenced B2R expression by siRNA and stimulated cells with 1 μM BK for 30 min. Western blot analysis showed that suppression of B2R inhibited the BK-mediated activation of ERK1/2, suggesting that BK induces ERK1/2 activation via B2R (Fig. 4B).

BK stimulates IL-6 production through the B2R-ERK pathway. BK treatment is able to induce the secretion of various cytokines such as IL-1β, IL-8 and IL-12 (12). Using real-time PCR and ELISA assay, we found that BK treatment (0.1-1 μM) increased the expression and secretion of IL-6 in the SW480 cells (Fig. 5A and B). We, then, silenced the expression of B2R by siRNA to analyze the effect of B2R on the BK-induced IL-6 production. We found that BK upregulated the expression and secretion of IL-6 in the control cells. However, after suppression of B2R by siRNA, the BK-mediated IL-6 production was greatly decreased (Fig. 5C and D). Furthermore, we pretreated SW480 cells with PD98059, a specific inhibitor of ERK1/2, to observe the function of the ERK pathway in the regulation of IL-6 production. The results showed that inactivation of ERK1/2 attenuated the BK-induced IL-6 production (Fig. 5E and F). Taken together, these data indicate that the B2R-ERK pathway is necessary for BK-induced IL-6 production.

IL-6 contributes to the BK-mediated invasion and migration of colorectal cancer cells. To reveal the role of IL-6 in invasion and migration, we silenced the expression of IL-6 in SW480 cells by siRNA (Fig. 6A). Then we subjected these cells to invasion and migration assays following BK treatment. We found that IL-6 suppression resulted in the inhibition of invasion and migration in the SW480 cells pretreated with BK, suggesting the involvement of IL-6 in the BK-mediated invasion and migration of colorectal cancer cells (Fig. 6B and C).

Discussion

As the most prominent member of the kinin group, bradykinin (BK) is an important player in the regulation of inflammation and cancer (13,14). Accumulating research shows that BK treatment can induce the activation of multiple intracellular pathways such as MAPK and PI3K/AKT signaling pathways in cancer cells (5,15), and thus contributes to the proliferation, angiogenesis and migration of cancer cells (16-18). Our present study demonstrated for the first time that BK treatment activated B2R and ERK1/2, which then led to an increase in IL-6 production, which finally promoted cell invasion and migration of colorectal cancer cells. These data strongly support the conclusion that BK is a potent stimulator of colorectal cancer cell invasion and migration.

BK exerts its effects via bradykinin receptors, which are categorized as B1R and B2R. B1R and B2R are both implicated in the progression of cancer. Studies have found that B1R and B2R are highly expressed in the cytoplasm of lung cancer, and B2R is overexpressed in head and neck squamous cell carcinomas (19,20). Using western blot analysis, we observed significant expression levels of B1R and B2R in all of the examined colorectal cancer cells, indicating a role of bradykinin receptors in colorectal cancer. It was also found that BK has a higher affinity to B2R as compared to B1R (12). Activation of B2R by BK promotes the growth and migration of prostate cancer cells (21,22). In the present study, we found that B2R suppression attenuated the BK-mediated invasion and migration of SW480 cells, whereas B1R suppression did not have an impact on the effect of BK on colorectal cancer cells. Thus, it appears that B2R, but not B1R, participates in regulating the BK-enhanced invasion and migration of colorectal cancer cells.

Numerous signaling pathways have been reported to be activated by BK, notably the MAPK, PKC and NF-κB pathways (11,23,24). Our results showed that BK treatment induced the activation of ERK1/2 through B2R. These findings suggest that the ERK pathway may contribute to the BK-mediated invasion and migration of colorectal cancer cells, as it is well known that the ERK pathway plays a pivotal role in colorectal cancer dissemination (25).

Of note, BK treatment is able to stimulate the generation of various cytokines such as IL-1 and tumor necrosis factor (TNF) (26). Studies have found that BK induces IL-6 secretion in lung fibroblasts (27). However, little is known concerning the effect of BK on IL-6 production in cancer cells. Here, we found that BK induced the expression and secretion of IL-6 via the B2R-ERK pathway. To the best of our knowledge, our findings are the first to show that BK treatment increases IL-6 production in colorectal cancer cells. IL-6 expression is frequently upregulated in various types of human cancers (28-30). Clinical data have shown that

IL-6 expression in tumor tissues correlates with lymph node metastasis of colorectal cancer (31), whereas experimental studies confirm that IL-6 promotes cell proliferation, invasion and metastasis of cancers (32-34). We found that suppression of IL-6 in SW480 cells decreased the BK-mediated invasion and migration, confirming that IL-6 plays an important role in colorectal cancer.

In conclusion, our study demonstrated that BK treatment enhanced the invasion and migration of colorectal cancer cells. Suppression of B2R attenuated the BK-mediated invasion and migration. The possible mechanism involved BK-stimulated B2R, subsequently leading to the activation of the ERK pathway and upregulation of IL-6 production. Therefore, it is likely that B2R may be a potential therapeutic target for the treatment of colorectal cancer.

Acknowledgements

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