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Bradykinin Enhances Cell Migration in Human Chondrosarcoma Cells Through BK Receptor Signaling Pathways

Wei-Hung Yang,[1,2,3] Jung-Tzu Chang,[4] Sheng-Feng Hsu,[5] Te-Mao Li,[5] Der-Yang Cho,[6] Chun-Yin Huang,[1] Yi-Chin Fong,[3,7**] and Chih-Hsin Tang[8*]

[1]*Department of Orthopaedic Surgery, China Medical University Beigang Hospital, Yun-Lin County, Taiwan*
[2]*Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan*
[3]*School of Chinese Medicine, China Medical University, Taichung, Taiwan*
[4]*Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan*
[5]*Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan*
[6]*Department of Neurosurgery, China Medical University Hospital, Taichung, Taiwan*
[7]*Department of Orthopaedic, China Medical University Hospital, Taichung, Taiwan*
[8]*Department of Pharmacology, China Medical University, Taichung, Taiwan*

ABSTRACT

Bradykinin (BK) is an inflammatory mediator, and shows elevated levels in regions of severe injury and inflammatory diseases. BK has recently been shown to be involved in carcinogenesis and cancer progression. In this study, we found that BK increased the migration and the expression of α2β1 integrin in human chondrosarcoma cells. We also found that human chondrosarcoma tissues had significantly higher expression of the B1 and B2 receptors comparing to normal cartilage. BK-mediated migration and integrin up-regulation was attenuated by B1 and B2 BK receptor siRNA or antagonist. Activations of phospholipase C (PLC), protein kinase Cδ (PKCδ), and NF-κB pathways after BK treatment was demonstrated, and BK-induced integrin expression and migration activity was inhibited by the specific inhibitor of PLC, PKCδ, and NF-κB cascades. Taken together, our results indicated that BK enhances the migration of chondrosarcoma cells by increasing α2β1 integrin expression through the BK receptors/PLC/PKCδ/NF-κB signal transduction pathway. J. Cell. Biochem. 109: 82–92, 2010.

KEY WORDS: BRADYKININ; CHONDROSARCOMA; BK RECEPTOR; INTEGRIN

Chondrosarcoma is the second most common malignancy of bone with a poor response to chemotherapy or radiation treatment currently used, making the management of chondrosarcomas a complicated challenge [Terek et al., 1998; Scully et al., 1999]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. In the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore a novel and adequate drug [Yuan et al., 2005].

Chondrosarcoma is a highly malignant tumor with a potent capacity to invade locally and distant metastasis [Berend et al., 1998; Schneiderbauer et al., 2004]. An approach to decreasing the ability of invasion and metastasis may facilitate the development of effective adjuvant therapy. The invasion of tumor cells is a complex, multistage process. To facilitate cell motility, invading cells need to change the cell-cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [Woodhouse et al., 1997]. Integrins are a family of

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*Correspondence to: Prof. Chih-Hsin Tang, Department of Pharmacology, College of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw

**Correspondence to: Yi-Chin Fong, Department of Orthopaedic, China Medical University Hospital, Taichung, Taiwan. E-mail: yichin.fong@mail.cmu.edu.tw

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transmembrane adhesion receptors comprising 19α and 8β subunits that interact noncovalently to form up to 24 different heterodimeric receptors [Giancotti and Ruoslahti, 1999; Humphries, 2000]. The combination of different integrin subunits on the cell surface allow cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen and vitronectin [Giancotti and Ruoslahti, 1999; Humphries, 2000]. Activation and elevated expression of integrin-coupled signaling effectors have been implicated in the induction of a wide variety of human cancers, including those of the breast, colon, prostate, and ovaries [White et al., 2004]. In addition, the expression of integrin has been shown to correlate with poor prognosis in cancers of the lung, pancreas and cutaneous melanoma [Bottger et al., 1999; Muller-Klingspor et al., 2001; Nikkola et al., 2004; Oshita et al., 2004; Yao et al., 2007]. Integrin has also been implicated in metastasis of lung, breast, bladder and colon cancers [Takenaka et al., 2000; Heyder et al., 2005; Seales et al., 2005].

Bradykinin (BK) is rapidly generated following inflammation or injury [Proud and Kaplan, 1988]. The release of BK is known to mediate multiple proinflammatory effects including smooth muscle contraction, vasodilation, increased vascular permeability, eicosanoid synthesis, and neuropeptide release [Proud and Kaplan, 1988; Hall, 1992]. The effects of BK are mediated via two G-protein-coupled receptors, B1 and B2, which have been pharmacologically characterized and defined by molecular cloning [Regoli and Barabe, 1980; Menke et al., 1994]. BK receptors have been implicated in tumorigenesis. For example, the B1 receptor was up-regulated in malignant prostate [Taub et al., 2003] and the B2 receptor was overexpressed in human gliomas [Zhao et al., 2005] and was detected in gastric, duodenal, lung, and hepatic cancers [Wu et al., 2002]. Most of the biological actions of BK are mediated through the B2 receptor, which signals via $G_{q/11}$ protein leading to an increase in $[Ca^{2+}]_i$ and protein kinase C (PKC) activation in different cell types [Enomoto et al., 1995; Ankorina-Stark et al., 1997; Wiernas et al., 1998]. However, the expression of BK receptors in various cancers has not been fully investigated, and the precise role of BK in the development and promotion of cancer remains unknown. We hypothesized that BK might be capable of regulating the migration of chondrosarcoma cells. In this study, we found that BK increased the migration and the expression of integrin in human chondrosarcoma cells. In addition, BK receptor, phospholipase C (PLC), PKCδ and NF-κB signaling pathways may be involved in increasing integrin expression and cell migration by BK.

MATERIALS AND METHODS

MATERIALS

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for IκBα, IKKαβ, p65, PLCβ3 and PKCδ, siRNA against B1, B2 BK receptor and PKCδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody specific for α2, α5, β3, α2β1, αvβ3, and α5β1 integrin were purchased from Chemicon (Temecula, CA). Rabbit polyclonal antibody specific for PLCβ3 phosphorylated at Ser^{437}, IKKαβ phosphorylated at $Ser^{180/181}$ and p65 phosphorylated at Ser^{276} were purchased from Cell Signaling and Neuroscience (Danvers, MA). U73122, GF109203X, Rottlerin, PDTC and TPCK were purchased from Calbiochem (La Jolla, CA). B1 receptor antagonist (Lys-(Leu8)des-Arg9-BK) and B2 receptor antagonist HOE140 were obtained from Tocris Bioscience (Ellisville, MO). The NF-κB luciferase plasmid was obtained from Stratagene (La Jolla, CA). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector, luciferase assay kit was obtained from Promega (Madison, MA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

The human chondrosarcoma cell line (JJ012) was kindly provided by Dr. Sean P Scully (University of Miami School of Medicine, Miami, FL). The cells were cultured in DMEM/α-MEM supplemented with 10% Fetal Bovine Serum (FBS) and maintained at 37°C in a humidified atmosphere of 5% CO_2.

PATIENTS AND SPECIMEN PREPARATION

Upon approval by the local ethics committee, specimens of tumor tissue or normal cartilage tissue were obtained from patients, who have been pathologically diagnosed with chondrosarcoma or knee osteoarthritis and have undergone surgical resection at the China Medical University Hospital. Tissue specimens were ground and then sonicated in a TRIzol kit. The mRNA level was analyzed using RT-PCR analysis.

MIGRATION ASSAY

The migration assay was performed using Transwell (Costar, NY; pore size, 8-μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the U73122, GF109203X, Rottlerin, PDTC, TPCK or vehicle control (0.1% DMSO). Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing BK were placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, and cells then were fixed in 1% formaldehyde for 5 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of the BK treatment (corrected number of invading cells = counted number of invading cells /percentage of viable cells) [Tan et al., 2009].

FLOW CYTOMETRIC ANALYSIS

Human chondrosarcoma cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After being rinsed in PBS, the cells were incubated with mouse anti-human antibody against integrin (1:100) for 1 h at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec. Inc.,

St. Louis, MO) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences) [Huang et al., 2007].

WESTERN BLOT ANALYSIS

The cellular lysates were prepared and proteins were then resolved on SDS–PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against PLC, PKCδ, p-IKK, IKK, p-p65 or p65 (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

PKC KINASE ACTIVITY ASSAY

PKC activity was assessed by PKC Kinase Activity Assay Kit according to manufacturer's instructions (Assay Designs, Ann Arbor, MI). PKC activity kit is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognized the phosphorylated form of the substrate.

QUANTITATIVE REAL-TIME PCR

The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). One hundred nanogram of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems). Quantitative RT-PCR assays were carried out in triplicate on StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the number of cycles at which the transcript was detected (denoted as C_T).

REPORTER ASSAY

The chondrosarcoma cells were transfected with reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were treated with inhibitors for 30 min, and then BK or vehicle was then added for another 24 h. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured [Yeh et al., 2008; Tang et al., 2008a].

STATISTICS

For statistical evaluation, Mann–Whitney *U*-test for non-Gaussian parameters and Student's *t*-test for Gaussian parameters (including Bonferroni correction). The difference is significant if the *P* value is <0.05

RESULTS

BK-DIRECTED CHONDROSARCOMA CELL MIGRATION INVOLVES α2β1 INTEGRIN UP-REGULATION

BK has been reported to stimulate migration and invasion of human cancer cells directionally [Wu et al., 2002; Taub et al., 2003]. The role of BK in chondrosarcoma cell migration was examined using the Transwell assay with correction of BK-induced proliferation effects on human chondrosarcoma cells [Tang et al., 2008b]. Figure 1A shows that BK enhanced the migration of human chondrosarcoma cells (JJ012 cells) in a dose-dependent manner. Previous studies have shown significant expression of integrins in human chondrosarcoma cells [Huang et al., 2007; Yeh et al., 2008]. We therefore, hypothesized that integrins may be involved in BK-directed chondrosarcoma cell migration. Flow cytometry analysis showed that BK induced the cell surface expression of α2β1 and α2 but not α5β1, αvβ3, β3 and α5 integrin in JJ012 cells (Fig. 1B). To confirm this finding, expression of mRNAs for the integrins in response to BK was analyzed by qPCR. Treatment of JJ012 cells with BK induced the mRNA expression of α2 and β1 integrins (Fig. 1C). Pretreatment of cells for 30 min with anti-α2β1 monoclonal antibody (mAb) (3 μg/ml) markedly inhibited the BK-induced cell migration (Fig. 1D). Therefore, the α2β1 integrin plays important role in BK-induced migration in human chondrosarcoma cells.

INVOLVEMENT OF BK RECEPTORS IN BK-MEDIATED MIGRATION OF CHONDROSARCOMA CELLS

BK exerts their effects through interaction with specific BK receptors (Bl and B2) [Regoli and Barabe, 1980; Menke et al., 1994]. However, little is known about the expression of B1 and B2 receptor in human chondrosarcoma cells. We examined human chondrosarcoma patients for the expression of the BK receptors using qPCR. Expression of mRNA levels of B1 and B2 in chondrosarcoma patients (Fig. 2A, lines 4–6) were significantly higher than those in normal cartilage (Fig. 2A, lines 1–3). Stimulation of cells with BK also increased the mRNA expression of B1 and B2 receptors (Fig. 2B). We next examined which BK subtype receptors were involved in the BK-mediated cell migration in chondrosarcoma cells. Transfection of JJ012 cells with B1 and B2 receptors siRNA reduced B1 and B2 protein expression, respectively (Fig. 2C). On the other hand, B1 and B2 receptors siRNA blocked BK-mediated cell migration (Fig. 2D). To confirm the role of BK receptors-dependent signaling in the regulation of cell migration in chondrosarcoma cells, cells were pretreated with the B1 receptor (Lys-(Leu8)des-Arg9-BK) and B2 receptor HOE140 antagonist reduced BK-mediated migration activity (Fig. 2E). In addition, B1 and B2 receptor antagonist also reduced BK-mediated integrin up-regulation (Fig. 2F). These data suggest that B1 and B2 BK receptors play a key role in migration of chondrosarcoma cells.

THE SIGNALING PATHWAYS OF PLC AND PKCδ ARE INVOLVED IN THE POTENTIATING ACTION OF BK STIMULATION

Previous studies have demonstrated that BK binds to BK receptors which induces the activation of PLC, triggering PI turnover and activation of PKC [Chen et al., 1995]. To study the intracellular

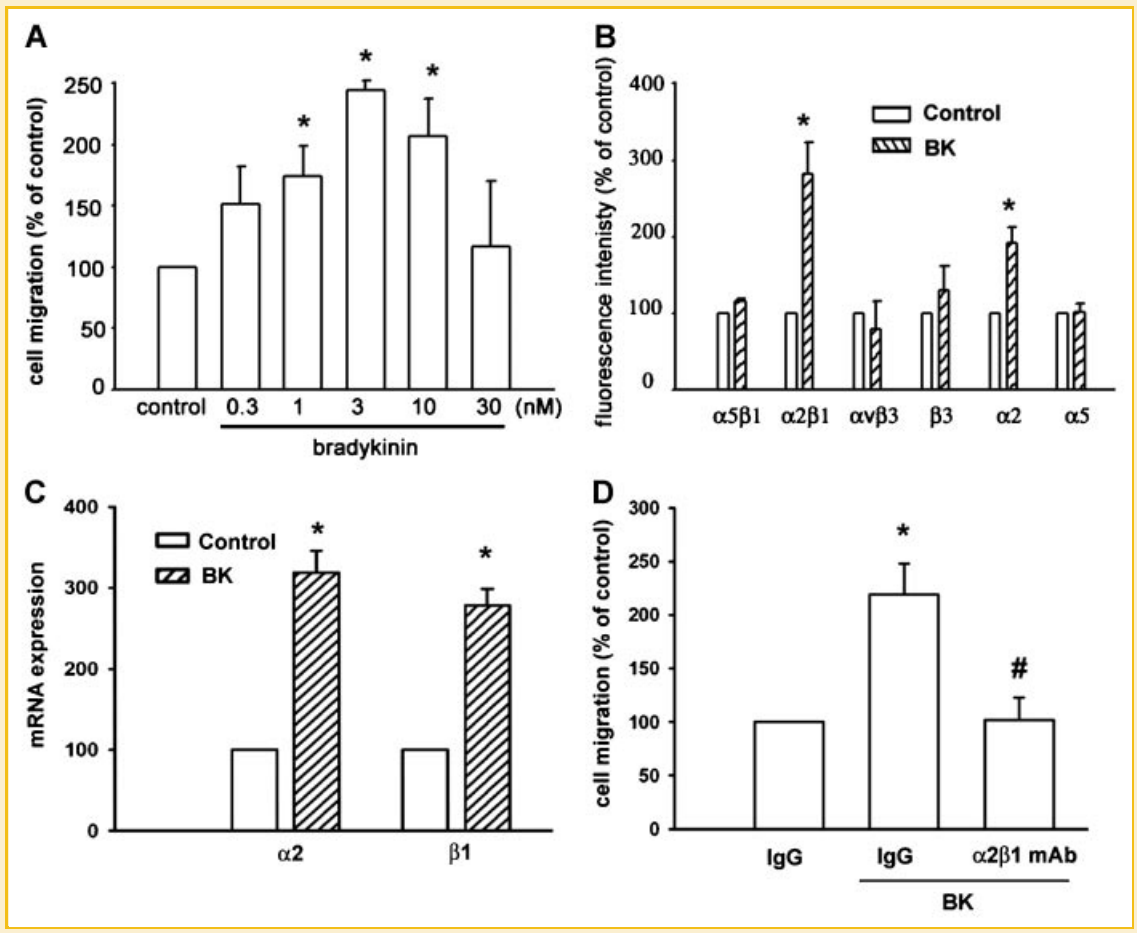

Fig. 1. BK-directed migration of human chondrosarcoma cells involves up-regulation of α2β1 integrins. JJ012 cells were incubated with various concentrations of BK, and in vitro migration activities measured with the Transwell after 24 h (A). JJ012 cells were incubated with BK (3 nM) for 24 h, and the cells surface integrin were determined using flow cytometry (B). Cells were incubated with BK (3 nM) for 24 h, and the mRNA levels of α2 and β1 integrin was determined using qPCR (C). Cells were pretreated with α2β1 monoclonal antibody (3 μg/ml), for 30 min followed by stimulation with BK. The in vitro migration activity measured after 24 h. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

signaling pathway involved in BK-induced cell migration, JJ012 cells were pretreated for 30 min with PI-PLC inhibitor, U73122. It was found that U73122 antagonized BK mediated cell migration and integrin expression (Fig. 3B,C). In addition, treatment of JJ012 cells with BK also induced the phosphorylation of PLCβ3 time-dependently (Fig. 3A). Transfection of cells with PLCβ siRNA reduced protein expression of PLCβ (Fig. 3B; upper panel). In addition, transfection of cells with PLCβ siRNA reduced BK-mediated cell migration and integrin up-regulation (Fig. 3B,C). As BK-induced cell migration was inhibited by U73122, indicating involvement of the PI-PLC pathway, which increases diacylglycerol levels leading to the activation of PKC. The PKC inhibitor GF109203X was thus used to examine whether PKC is involved in the action of BK. Pretreatment with GF109203X inhibited the enhancement effect of BK (Fig. 4A). It has been reported that PKCδ activation is implicated in the control of MMP and IL-6 expression via BK [Hsieh et al., 2008; Lee et al., 2008]. To further explore whether PKCδ might plays a crucial role in BK-induced cell migration and integrin expression, the selective PKCδ inhibitor, rottlerin, was used. It was demonstrated that treatment with rottlerin antagonized BK mediated cell migration and integrin expression (Fig. 4D,E). Stimulation of cells with BK also increased PKCδ phosphoylation and kinase activity time-dependently (Fig. 4B,C). Transfection of cells with PKCδ siRNA reduced protein expression of PLCδ (Fig. 4D; upper panel). In addition, transfection of cells with PKCδ siRNA reduced BK-mediated cell migration and integrin up-regulation (Fig. 4D,E). Based on these results, it appears that BK acts through the BK receptors, PI-PLCβ3- and PKCδ-dependent signaling pathway to enhance cell migration and integrin up-regulation in human chondrosarcoma cells.

NF-κB SIGNALING PATHWAY IS INVOLVED IN BK-MEDIATED INTEGRIN UP-REGULATION AND MIGRATION ACTIVITY

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [Boukerche et al., 2007; Tang et al., 2008b]. To examine whether NF-κB activation is

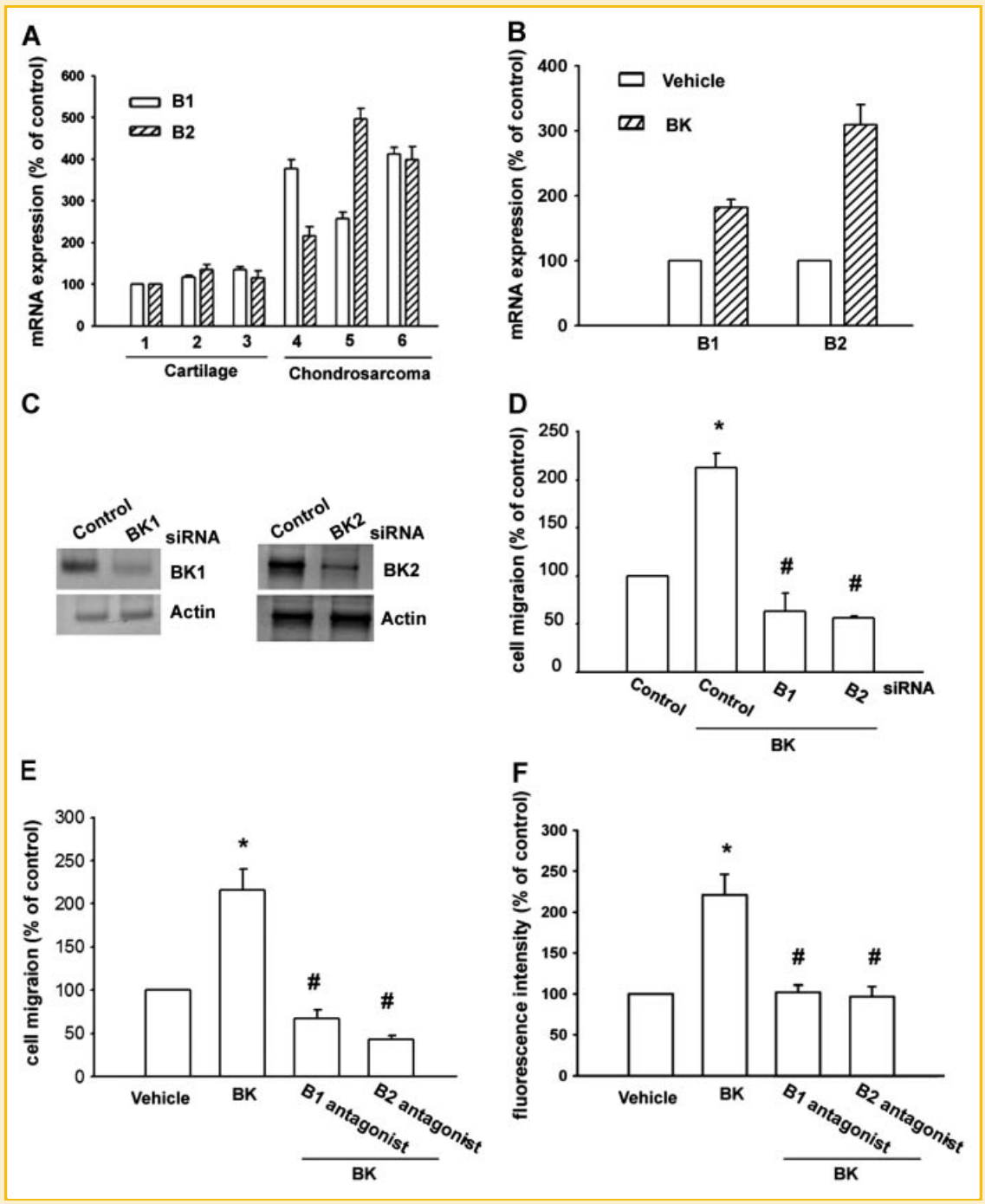

Fig. 2. BK receptors are involved in BK-mediated migration of human chondrosarcoma cells. Total RNA were extracted from normal cartilage (lines 1–3) or from chondrosarcoma patients (lines 4–6) and subjected to qPCR analysis for B1 and B2 BK receptors (A). JJ012 cells were incubated with BK (3 nM) for 24 h, and the mRNA levels of B1 and B2 receptors was determined using qPCR (B). JJ012 cells were transfected with B1, B2 or control siRNA for 24 h, and the expression of BK receptors was examined by Western blot analysis (C). JJ012 cells were transfected with B1, B2 or control siRNA for 24 h followed by stimulation with BK (3 nM), and in vitro migration was measured with the Transwell after 24 h (D). Cells were pretreated for 30 min with B1 antagonist (10 μM) or B2 antagonist (10 nM) followed by stimulation with BK (3 nM), and in vitro migration and cells surface $\alpha 2\beta 1$ integrin were measured by Transwell and flow cytometry (E,F). Results are expressed as the mean $\pm$ SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

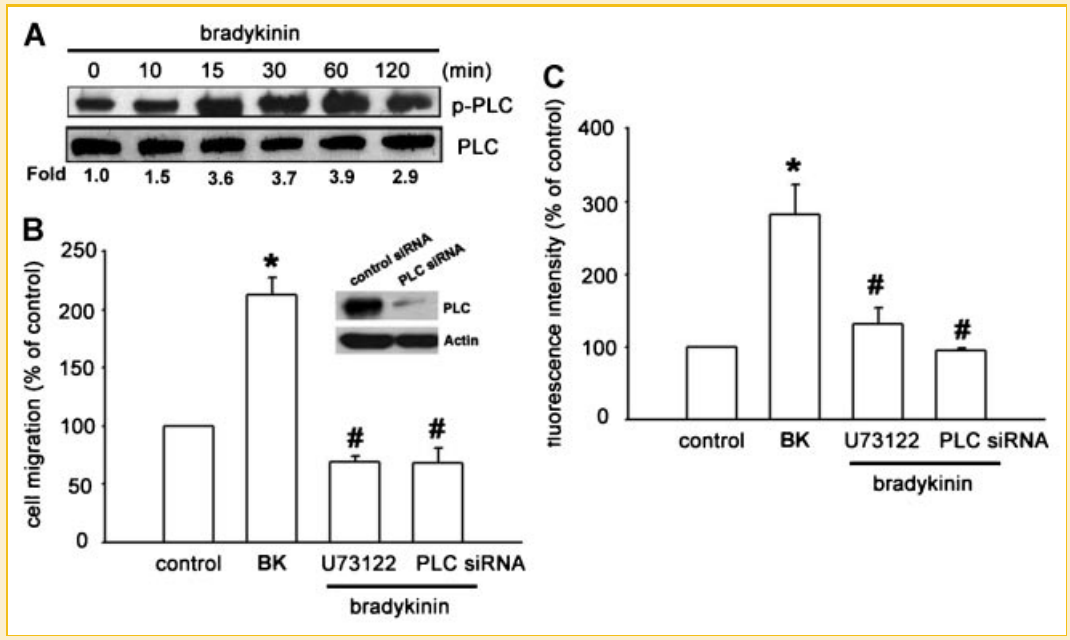

Fig. 3. PLC pathway is involved in BK-mediated migration and integrin up-regulation in human chondrosarcoma cells. JJ012 cells were incubated with BK (3 nM) for various time intervals, and PLCβ3 phosphorylation was determined by Western blot (A). JJ012 cells were transfected with PLCβ3 or control siRNA for 24 h, the Western blot analysis showed the inhibitory effect of PLCβ3 siRNA on the protein expression of PLCβ3 (B; upper panel). Cells were pretreated with U73122 (3 μM) or transfected with PLCβ3 siRNA for 24 h followed by stimulation with BK, and in vitro migration (B) and cells surface α2β1 integrin (C) were measured with the Transwell and flow cytometry after 24 h. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

involved in BK-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Figure 5A shows that chondrosarcoma cells pretreated with PDTC (10 μM) inhibited the BK-induced migration of chondrosarcoma cells. Furthermore, cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced BK-induced migration of cancer cells (Fig. 5A). In addition, treatment of cells with PDTC or TPCK also antagonized BK-induced expression of α2β1 integrin (Fig. 5B). Therefore, involvement of NF-κB pathway in BK induced migration of chondrosarcoma cells. We further examined the upstream molecules involved in BK-induced NF-κB activation. Stimulation of cells with BK induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6A). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the BK-induced cell migration and integrin up-regulation (Fig. 6B,C). These data suggest that IKKα/β activation is involved in BK-induced the migration activity of human chondrosarcoma cells. Treatment of chondrosarcoma cells with BK also caused IκBα phosphorylation in a time-dependent manner (Fig. 6A). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [Madrid et al., 2001], and the antibody specific against phosphorylated p65 Ser^{536} was employed to examine p65 phosphorylation. Treatment of cells with BK for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 6A). To directly determine NF-κB activation after BK treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Figure 6D, BK treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity. In addition, U73122, rottlerin, PDTC and TPCK reduced BK-mediated NF-κB activity (Fig. 6D). Moreover, co-transfection of cells with PLC and PKC siRNA or IKKα and IKKβ mutant also abolished BK-induced NF-κB promoter activity (Fig. 6E,F). Pretreatment of cells with U73122 and rottlerin reduced BK-mediated p65 phosphorylation (Fig. 6G). Taken together, these data suggest that activation of BK receptors, PLC and PKCδ pathway is required for BK-induced NF-κB activation in chondrosarcoma cells.

DISCUSSION

It is estimated that chondrosarcoma accounts for 3.6% of the annual incidence of all primary bone malignancies in USA, after multiple myeloma and osteogenic sarcoma [Schneiderbauer et al., 2004]. The distant metastatic potential of chondrosarcoma has previously been reported [Berend et al., 1998; Schneiderbauer et al., 2004]. Patients who develop metastatic chondrosarcoma has poor prognosis [Klein, 2009]. Although past research has shown that BK and BK receptors expression enhanced tumorigenesis and metastasis of human cancer cells, its role in chondrosarcoma invasion was not elucidated [Enomoto et al., 1995; Molina et al., 2009]. This study shows for the first time that BK mediates migration of human chondrosarcoma cells. BK mediated α2β1 integrin expression and cell migration through BK receptor, PLC, PKCδ and NF-κB pathways.

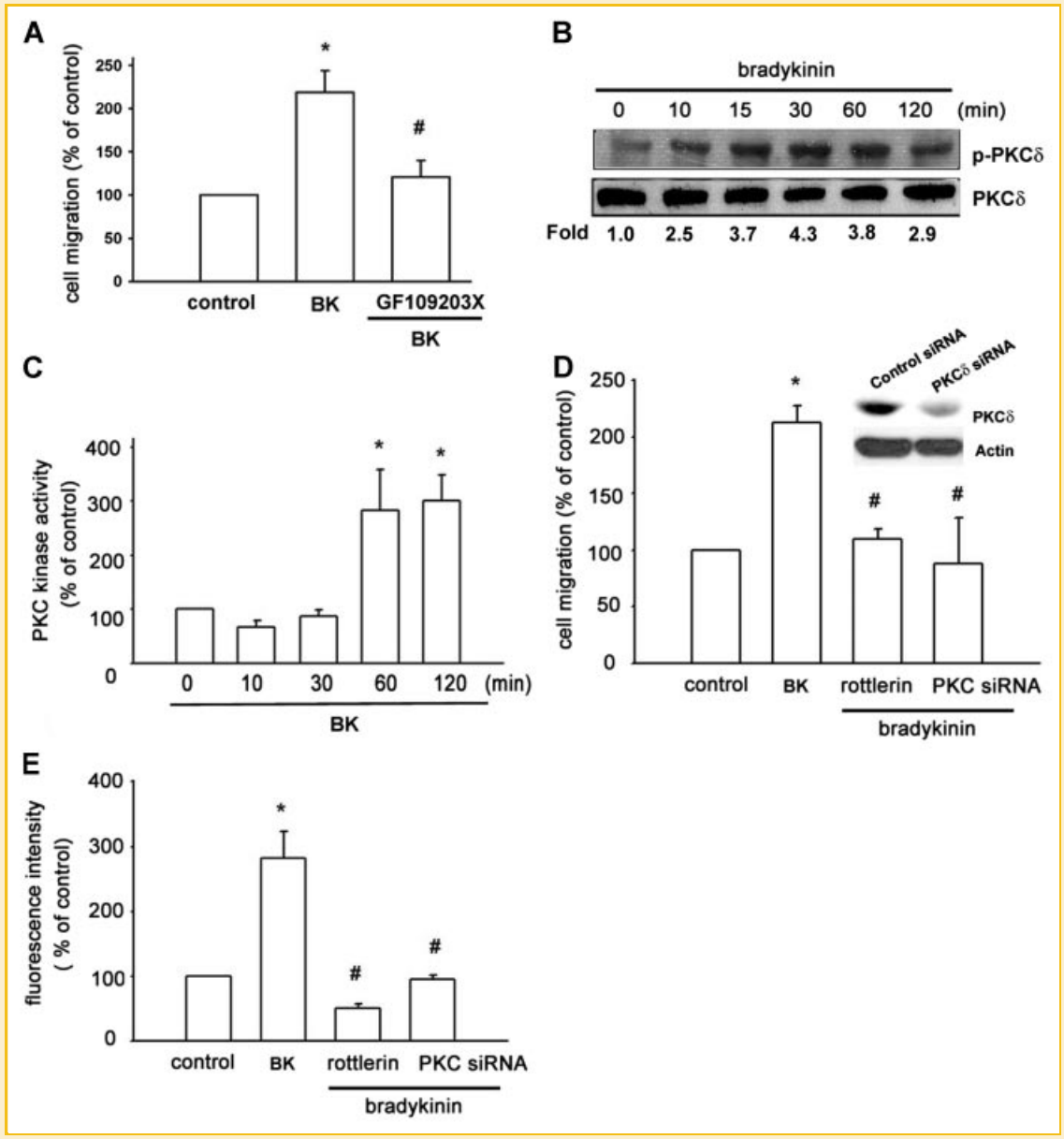

Fig. 4. PKCδ pathway is involved in BK-mediated migration and integrin up-regulation in human chondrosarcoma cells. Cells were pretreated with GF109203X (3 μM), for 30 min followed by stimulation with BK (3 nM). The in vitro migration activity measured after 24 h (A). JJ012 cells were incubated with BK (3 nM) for various time intervals, and PKCδ phosphorylation and kinase activity were determined by Western blot and ELISA kit (B,C). JJ012 cells were transfected with PKCδ or control siRNA for 24 h, the Western blot analysis showed the inhibitory effect of PKCδ siRNA on the protein expression of PKCδ (D; upper panel). Cells were pretreated with rottlerin (3 μM) or transfected with PKCδ siRNA for 24 h followed by stimulation with BK (3 nM), and in vitro migration (D) and cells surface $\alpha 2\beta 1$ integrin (E) were measured with the Transwell and flow cytometry after 24 h. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

Two types of BK receptors have been defined and cloned: B1 and B2 BK receptors [Hall, 1997]. However, the expression of the BK receptor isoforms in human chondrosarcoma cells are mostly unknown. Using qPCR analysis, we found that primary chondrosarcoma cells express both B1 and B2 BK receptor isoforms. It has been reported that B1 is almost absent but is increased in pathological conditions [Wu et al., 2002; Taub et al., 2003]. Our data also confirm these results that B1 receptor increased in pathological condition of chondrosarcoma. Previous reported that B2 receptor is activated by BK, the B1 receptor is activated by des-Arg-BK [Su, 2006; Chen and Johnson, 2007]. In this study, BK induced cell migration and integrin up-regulation was reduced by B1 and B2 receptor-specific antagonist and siRNA. Therefore, B1 receptor also involved in BK-mediated cell functions. The similar

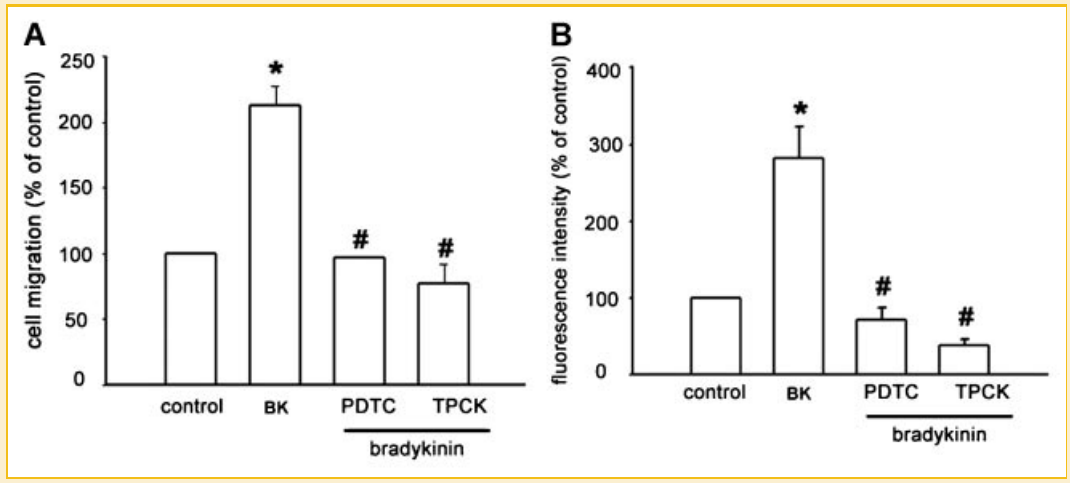

Fig. 5. BK induces cells migration and integrin up-regulation through NF-κB. A: Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with BK (3 nM), and in vitro migration was measured with the Transwell after 24 h. B: Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with BK for 24 h, and the cell surface α2β1 integrin was measured by flow cytometry. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

function has also been reported in the migration of microglial, which involved BK-induced B1 receptor signaling [Ifuku et al., 2007]. In this reported, the inhibition of either of the BK receptors produced the same effects on migration and integrin expression. These data indicated that BK receptors were compensative the inhibition of BK receptors. It has been postulated by Mareau that while BK increases the expression of B1 receptor, it decreases the expression of B2 receptor [Marceau and Regoli, 2004]. In our study, treatment of BK only slightly increased B1 receptor expression (~1.7-fold). Furthermore, BK strongly increased B2 receptor (~3.0-fold). Therefore, B2 receptor is much more important in BK-induced motility, although the role of B1 receptor cannot be ruled out.

Integrins link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth and differentiation [Giancotti and Ruoslahti, 1999; Humphries, 2000]. Using flow cytometry analysis, we found that BK increased α2β1 but not α5β1, αvβ3, β3, and α5 integrin expression, which plays an important role during tumor metastasis. Furthermore, BK also increased the mRNA levels of α2 and β2 integrins. In the present study, we used α2β1 integrin antibody to determine the role of α2β1 integrin and found that it inhibited BK-induced cancer migration. Therefore, α2β1 integrin is mostly important in BK-mediated induction of cancer migration.

Several isoforms of PKC have been characterized at the molecular level and these have been found to mediate several cellular molecular responses [Steinberg, 2008]. We demonstrated that PKC inhibitor GF109203X antagonized the BK-mediated enhancement of cell migration, suggesting that PKC activation is an obligatory event in BK-induced migration activity in these cells. In addition, rottlerin (a specific PKCδ inhibitor) also inhibited BK-induced cell migration and integrin up-regulation. This was further confirmed by the result that the PKCδ siRNA inhibited the enhancement of cell migration and integrin up-regulation by BK. Incubation of cells with BK also increase PKCδ phosphorylation and kinase activity. To examine whether the others PKC isoforms are involved in BK-mediated migration, the specific siRNA were used. We also found that transfection of cells with PKCα, PKCβ or PKCε siRNA did not affect BK-mediated cell migration (Supplemental Data, Fig. S1). Therefore, PKCδ is major PKC isoform in BK-mediated migration activity and integrin expression. Whether other PKC isoforms are involved needs further investigation. It has been reported that BK stimulates PLC and results in the activation of PKC [Chen et al., 1995; Lee et al., 2008]. The PI-PLC inhibitor U73122 inhibited the increase in cell migration by BK, suggesting that the PI-PLC pathway is involved in PKC activation by BK. In addition, BK also increased the phosphorylation of PLCβ3. These data suggest the BK receptors, PLC and PKCδ pathway is required for BK-induced cell migration and integrin up-regulation. To examine whether the others PLC are involved in BK-mediated migration, the specific siRNA was used. We also found that transfection of cells with PLCγ siRNA did not affect BK-mediated cell migration (Supplemental Data, Fig. S1). Therefore, PLCβ is major PLC isoform in BK-mediated migration activity and integrin expression. Whether other PLC isoforms are involved needs further investigation.

The prognosis of patients with chondrosarcoma distant metastasis is generally considered very poor, hence, preventing human chondrosarcoma metastasis is an important issue nowadays. Our study observes that BK increases the expression of α2β1 integrin via the BK receptors, PLC, PKCδ, IKKα/β, and NF-κB-dependent pathway and enhances migration of human chondrosarcoma cells. Furthermore, the discovery of BK-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

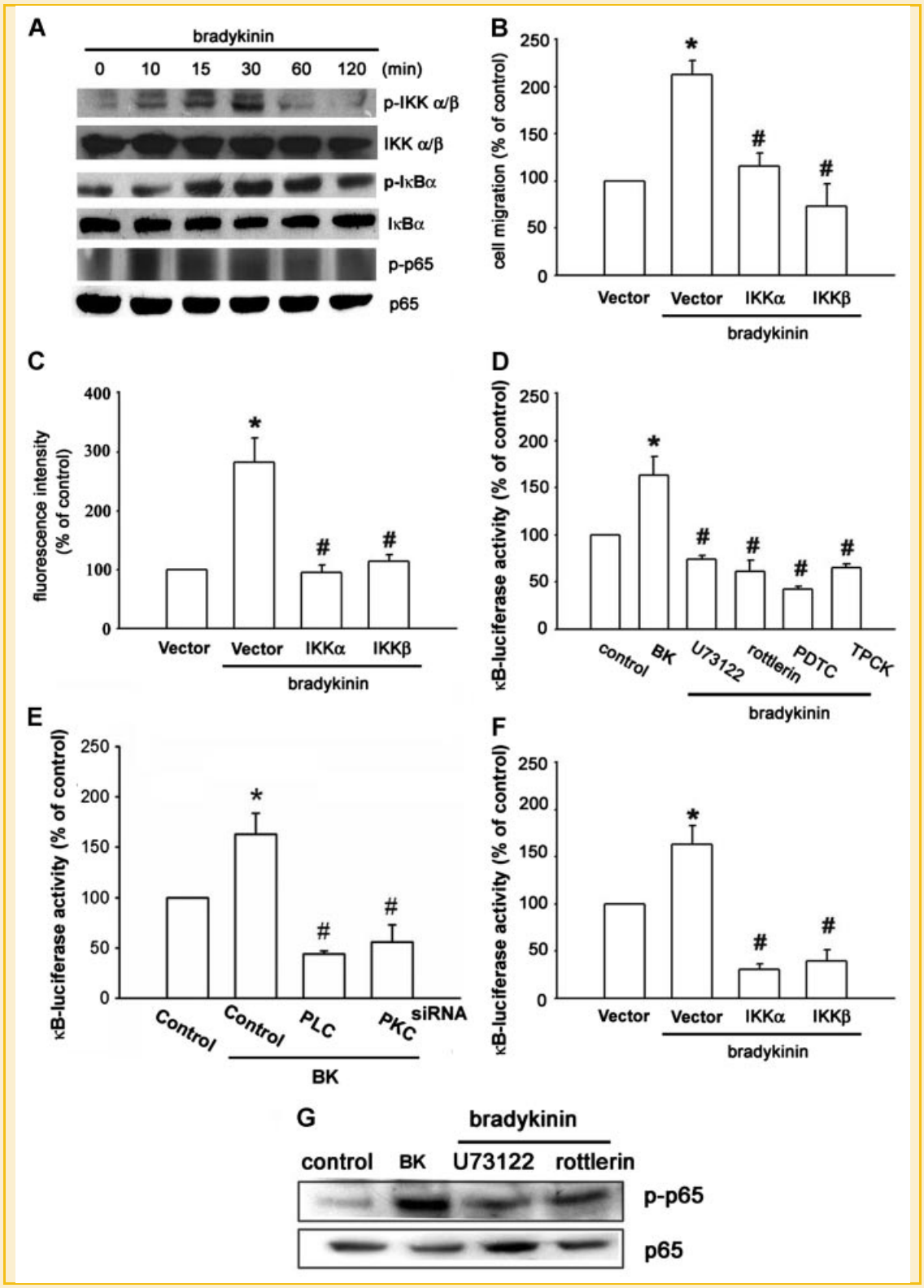

Fig. 6. IKK and p65 activation are involved in BK-mediated cell migration in human chondrosarcoma cells. JJ012 cells were incubated with BK (3 nM) for indicated time intervals, and p-IKKα/β, p-IκBα and p-p65 expression was determined by Western blot analysis (A). JJ012 cells were transfected with dominant negative mutant of IKKα or IKKβ for 24 h followed by stimulation with BK (3 nM), and in vitro migration (B) and cells surface α2β1 integrin (C) were measured with the Transwell and flow cytometry after 24 h. JJ012 cells transiently transfected with κB-luciferase plasmid for 24 h and then pretreated with U73122, rottlerin, PDTC and TPCK for 30 min (D) or co-transfection with PLC and PKC siRNA (E) or IKKα and IKKβ mutant (F), before incubation with BK (3 nM) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Cells were pretreated with U73122 or rottlerin for 30 min followed by stimulation with BK (3 nM) for 60 min, and p-p65 expression was examined by Western blot analysis (G). Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with BK-treated group.

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REFERENCES

Ankorina-Stark I, Haxelmans S, Schlatter E. 1997. Receptors for bradykinin and prostaglandin E2 coupled to Ca2+ signalling in rat cortical collecting duct. Cell Calcium 22:269–275.

Berend KR, Toth AP, Harrelson JM, Layfield LJ, Hey LA, Scully SP. 1998. Association between ratio of matrix metalloproteinase-1 to tissue inhibitor of metalloproteinase-1 and local recurrence, metastasis, and survival in human chondrosarcoma. J Bone Joint Surg Am 80:11–17.

Bottger TC, Maschek H, Lobo M, Gottwohl RG, Brenner W, Junginger T. 1999. Prognostic value of immunohistochemical expression of beta-1 integrin in pancreatic carcinoma. Oncology 56:308–313.

Boukerche H, Su ZZ, Emdad L, Sarkar D, Fisher PB. 2007. mda-9/Syntenin regulates the metastatic phenotype in human melanoma cells by activating nuclear factor-kappaB. Cancer Res 67:1812–1822.

Chen JJ, Johnson EJ. 2007. Targeting the bradykinin B1 receptor to reduce pain. Expert Opin Ther Targets 11:21–35.

Chen CC, Chang J, Chen WC. 1995. Role of protein kinase C subtypes alpha and delta in the regulation of bradykinin-stimulated phosphoinositide breakdown in astrocytes. Mol Pharmacol 48:39–47.

Enomoto K, Furuya K, Yamagishi S, Oka T, Maeno T. 1995. Release of arachidonic acid via Ca2+ increase stimulated by pyrophosphonucleotides and bradykinin in mammary tumour cells. Cell Biochem Funct 13:279–286.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285:1028–1032.

Hall JM. 1992. Bradykinin receptors: Pharmacological properties and biological roles. Pharmacol Ther 56:131–190.

Hall JM. 1997. Bradykinin receptors. Gen Pharmacol 28:1–6.

Heyder C, Gloria-Maercker E, Hatzmann W, Niggemann B, Zanker KS, Dittmar T. 2005. Role of the beta1-integrin subunit in the adhesion, extravasation and migration of T24 human bladder carcinoma cells. Clin Exp Metastasis 22:99–106.

Hsieh HL, Wu CY, Yang CM. 2008. Bradykinin induces matrix metalloproteinase-9 expression and cell migration through a PKC-delta-dependent ERK/Elk-1 pathway in astrocytes. Glia 56:619–632.

Huang YC, Hsiao YC, Chen YJ, Wei YY, Lai TH, Tang CH. 2007. Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-kappaB-dependent pathway in human lung cancer cells. Biochem Pharmacol 74:1702–1712.

Humphries MJ. 2000. Integrin structure. Biochem Soc Trans 28:311–339.

Ifuku M, Farber K, Okuno Y, Yamakawa Y, Miyamoto T, Nolte C, Merrino VF, Kita S, Iwamoto T, Komuro I, Wang B, Cheung G, Ishikawa E, Ooboshi H, Bader M, Wada K, Kettenmann H, Noda M. 2007. Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger. J Neurosci 27:13065–13073.

Klein CA. 2009. Parallel progression of primary tumours and metastases. Nat Rev Cancer 9:302–312.

Lee CH, Shieh DC, Tzeng CY, Chen CP, Wang SP, Chiu YC, Huang CY, Hsu CJ, Fong YC, Tang CH. 2008. Bradykinin-induced IL-6 expression through bradykinin B2 receptor, phospholipase C, protein kinase Cdelta and NF-kappaB pathway in human synovial fibroblasts. Mol Immunol 45:3693–3702.

Madrid LV, Mayo MW, Reuther JY, Baldwin AS, Jr. 2001. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem 276:18934–18940.

Marceau F, Regoli D. 2004. Bradykinin receptor ligands: Therapeutic perspectives. Nat Rev Drug Discov 3:845–852.

Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck KA, Ransom RW, Strader CD, Linemeyer DL, Hess JF. 1994. Expression cloning of a human B1 bradykinin receptor. J Biol Chem 269:21583–21586.

Molina L, Matus CE, Astroza A, Pavicic F, Tapia E, Toledo C, Perez JA, Nualart F, Gonzalez CB, Burgos RA, Figueroa CD, Ehrenfeld P, Poblete MT. 2009. Stimulation of the bradykinin B(1) receptor induces the proliferation of estrogen-sensitive breast cancer cells and activates the ERK1/2 signaling pathway. Breast Cancer Res Treat (in press). DOI: 10.1007/s10549-009-0314-4.

Muller-Klingspor V, Hefler L, Obermair A, Kaider A, Breitenecker G, Leodolte S, Kohlberger P. 2001. Prognostic value of beta1-integrin (=CD29) in serous adenocarcinomas of the ovary. Anticancer Res 21:2185–2188.

Nikkola J, Vihinen P, Vlaykova T, Hahka-Kemppinen M, Heino J, Pyrhonen S. 2004. Integrin chains beta1 and alphav as prognostic factors in human metastatic melanoma. Melanoma Res 14:29–37.

Oshita F, Kameda Y, Hamanaka N, Saito H, Yamada K, Noda K, Mitsuda A. 2004. High expression of integrin beta1 and p53 is a greater poor prognostic factor than clinical stage in small-cell lung cancer. Am J Clin Oncol 27:215–219.

Proud D, Kaplan AP. 1988. Kinin formation: Mechanisms and role in inflammatory disorders. Annu Rev Immunol 6:49–83.

Regoli D, Barabe J. 1980. Pharmacology of bradykinin and related kinins. Pharmacol Rev 32:1–46.

Schneiderbauer MM, Blanchard C, Gullerud R, Harmsen WS, Rock MG, Shives TC, Sim FH, Scully SP. 2004. Scapular chondrosarcomas have high rates of local recurrence and metastasis. Clin Orthop Relat Res 426:232–238.

Scully SP, Berend KR, Qi WN, Harrelson JM. 1999. Collagenase specificity in chondrosarcoma metastasis. Braz J Med Biol Res 32:885–889.

Seales EC, Jurado GA, Brunson BA, Wakefield JK, Frost AR, Bellis SL. 2005. Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. Cancer Res 65:4645–4652.

Steinberg SF. 2008. Structural basis of protein kinase C isoform function. Physiol Rev 88:1341–1378.

Su JB. 2006. Kinins and cardiovascular diseases. Curr Pharm Des 12:3423–3435.

Takenaka K, Shibuya M, Takeda Y, Hibino S, Gemma A, Ono Y, Kudoh S. 2000. Altered expression and function of beta1 integrins in a highly metastatic human lung adenocarcinoma cell line. Int J Oncol 17:1187–1194.

Tan TW, Yang WH, Lin YT, Hsu SF, Li TM, Kao ST, Chen WC, Fong YC, Tang CH. 2009. Cyr61 increases migration and MMP-13 expression via alphav-beta3 integrin, FAK, ERK and AP-1-dependent pathway in human chondrosarcoma cells. Carcinogenesis 30:258–268.

Tang CH, Chuang JY, Fong YC, Maa MC, Way TD, Hung CH. 2008a. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-kappaB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis 29:1483–1492.

Tang CH, Tan TW, Fu WM, Yang RS. 2008b. Involvement of matrix metalloproteinase-9 in stromal cell-derived factor-1/CXCR4 pathway of lung cancer metastasis. Carcinogenesis 29:35–43.

Taub JS, Guo R, Leeb-Lundberg LM, Madden JF, Daaka Y. 2003. Bradykinin receptor subtype 1 expression and function in prostate cancer. Cancer Res 63:2037–2041.

Terek RM, Schwartz GK, Devaney K, Glantz L, Mak S, Healey JH, Albino AP. 1998. Chemotherapy and P-glycoprotein expression in chondrosarcoma. J Orthop Res 16:585–590.

White DE, Kurpios NA, Zuo D, Hassell JA, Blaess S, Mueller U, Muller WJ. 2004. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 6:159–170.

Wiernas TK, Davis TL, Griffin BW, Sharif NA. 1998. Effects of bradykinin on signal transduction, cell proliferation, and cytokine, prostaglandin E2 and collagenase-1 release from human corneal epithelial cells. Br J Pharmacol 123:1127–1137.

Woodhouse EC, Chuaqui RF, Liotta LA. 1997. General mechanisms of metastasis. Cancer 80:1529–1537.

Wu J, Akaike T, Hayashida K, Miyamoto Y, Nakagawa T, Miyakawa K, Muller-Esterl W, Maeda H. 2002. Identification of bradykinin receptors in clinical cancer specimens and murine tumor tissues. Int J Cancer 98:29–35.

Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D, Park C. 2007. Increased beta1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res 67:659–664.

Yeh YY, Chiao CC, Kuo WY, Hsiao YC, Chen YJ, Wei YY, Lai TH, Fong YC, Tang CH. 2008. TGF-beta1 increases motility and alphavbeta3 integrin up-regulation via PI3K, Akt and NF-kappaB-dependent pathway in human chondrosarcoma cells. Biochem Pharmacol 75:1292–1301.

Yuan J, Dutton CM, Scully SP. 2005. RNAi mediated MMP-1 silencing inhibits human chondrosarcoma invasion. J Orthop Res 23:1467–1474.

Zhao Y, Xue Y, Liu Y, Fu W, Jiang N, An P, Wang P, Yang Z, Wang Y. 2005. Study of correlation between expression of bradykinin B2 receptor and pathological grade in human gliomas. Br J Neurosurg 19:322–326.