ARTICLE

Involvement of Integrin Up-Regulation in RANKL/RANK Pathway of Chondrosarcomas Migration

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ABSTRACT

Invasion of tumor cells is the primary cause of therapeutic failure in malignant chondrosarcomas treatment. Receptor activator of nuclear factor-κB ligand (RANKL) and its receptor, RANK, play a key roles in osteoclastogenesis and tumor metastasis. We found that the RANKL and RANK expression in human chondrosarcoma tissues was higher than that in normal cartilage. We also found that RANKL directed the migration and increased cell surface expression of β1 integrin in human chondrosarcoma cells (JJ012 cells). Pretreatment of JJ012 cells with MAPK kinase (MEK) inhibitors, PD98059 or U0126, inhibited the RANKL-induced migration and integrin expression. Stimulation of cells with RANKL increased the phosphorylation of MEK and extracellular signal-regulating kinase (ERK). In addition, NF-κB inhibitor (PDTC) or IκB protease inhibitor (TPCK) also inhibited RANKL-induced cells migration and integrin up-regulation. Taken together, these results suggest that the RANKL acts through MEK/ERK, which in turn activates IKKα/β and NF-κB, resulting in the activation of β1 integrin and contributing to the migration of human chondrosarcoma cells. J. Cell. Biochem. 111: 138–147, 2010.   © 2010 Wiley-Liss, Inc.

KEY WORDS: RANKL; RANK; MIGRATION; CHONDROSARCOMA; ERK

Chondrosarcoma is a malignant primary bone tumor with a poor response to currently used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge [Terek et al., 1998]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. Due to 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 remedy [Yuan et al., 2005].

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors [Gupta and Massague, 2006]. The invasion of tumor cells is a complex, multistage process. To facilitate the 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 transmembrane adhesion receptors comprising 19α and 8β subunits that interact noncovalently to form up to 24 different heterodimeric receptors. The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen, and vitronectin [Humphries, 2000]. Because integrins are the primary receptors for cellular adhesion to ECM molecules, they act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration, and tissue remodeling [Stupack, 2007]. 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, 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].

Receptor activator of nuclear factor-κB ligand [RANK ligand (RANKL)] is a tumor necrosis factor ligand superfamily member that is essential for osteoclast formation, function, and survival [Boyle et al., 2003]. RANKL interacts with the cell surface receptor RANK and in turn recruits TNFR associated factors (TRAF)1, 2, 3, 5, and 6 [Darnay et al., 1998]. The receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to NF-κB activation, and recruitment of TRAF2 leads to c-Jun N-terminal kinase (JNK) activation [Lee et al., 1997; Darnay et al., 1999]. RANKL is also known for activating NF-κB, JNK, and p38 and p44/p42 mitogen-activated protein kinase (MAPK) [Lee et al., 1997; Darnay et al., 1999]. However, the mechanism of this cytokine involved in tumor metastasis is still not fully understood.

Previous studies have shown that RANKL modulates cell migration and invasion in human cancer cells [Armstrong et al., 2008; Heymann et al., 2008; Virk et al., 2009]. Meanwhile, interaction of RANKL with its specific receptor RANK on the surface of cancer cells has been suggested to induce cancer invasion [Armstrong et al., 2008; Heymann et al., 2008]. However, the effect of RANKL and RANK receptor on integrin expression and migration activity in human chondrosarcoma cells is mostly unknown. In this study, we explored whether RANKL and RANK interaction increased the migration and integrin expression of in human chondrosarcoma cells. In addition, MAPK kinase (MEK), ERK, IKKα/β and NF-κB signaling pathways may be involved in the increase of integrin expression and cells migration by RANKL.

MATERIALS AND METHODS

MATERIALS

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-MEK, MEK, p-ERK, ERK, IKKα/β, IκBα, p-IκBα, p65, α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody specific for α2, α5, β1, β3, and αvβ3 integrin was purchased from Chemicon (Temecula, CA). U0126, PD98059, TPCK, and PDTC were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for phosphor-IKKα/β (Ser$^{180/181}$) and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA). The recombinant human RANKL was purchased from PeproTech (Rocky Hill, NJ). Mouse monoclonal antibody specific for RANKL (RANKL-Fc) and osteoprotegerin (OPG) were purchased from R&D Systems (Minneapolis, MN). The MEK1 dominant-negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The ERK2 dominant-negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

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

MIGRATION ASSAY

The migration assay was performed by using Transwell (Costar, NY; pore size, 8-μm) in 24-well dishes. Before migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the U0126, PD98059, PDTC, TPCK, or vehicle control (0.1% DMSO). The concentrations of inhibitors did not induce cell death of JJ012 cells confirmed by cell viability assay (data not shown). 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 RANKL was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, then cells were fixed in methanol for 15 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 RANKL treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) [Fong et al., 2008].

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).

WESTERN BLOT ANALYSIS

The cellular lysates were prepared as described previously [Tang et al., 2008]. Proteins were 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 IκBα, p-IκB, or IKKα/β (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 by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The activities of ERK were determined using a kit from Cell Signaling Technology according to the manufacturer's instructions.

QUANTITATIVE REAL TIME PCR

Total RNA was extracted from chondrosarcomas by using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). Two micrograms of total RNA was

reverse transcribed into cDNA by using oligo(dT) primer. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). One hundred nanograms 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). qPCR assays were carried out in triplicate on an 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 cycle number at which the transcript was detected (denoted C_T).

PATIENTS AND SPECIMEN PREPARATION

After approval by the local ethics committee, specimens of tumor tissue were obtained from patients, who had been pathologically diagnosed with chondrosarcoma and had undergone surgical resection at the China Medical University Hospital. Tissue specimens were ground and then sonicated in a Trizol. The mRNA level was analyzed by using qPCR analysis.

TRANSFECTION AND REPORTER GENE ASSAY

Human chondrosarcoma cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. JJ012 cells were grown to 80% confluence in 12-well plates and were transfected on the following day with Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA). DNA and LF2000 were
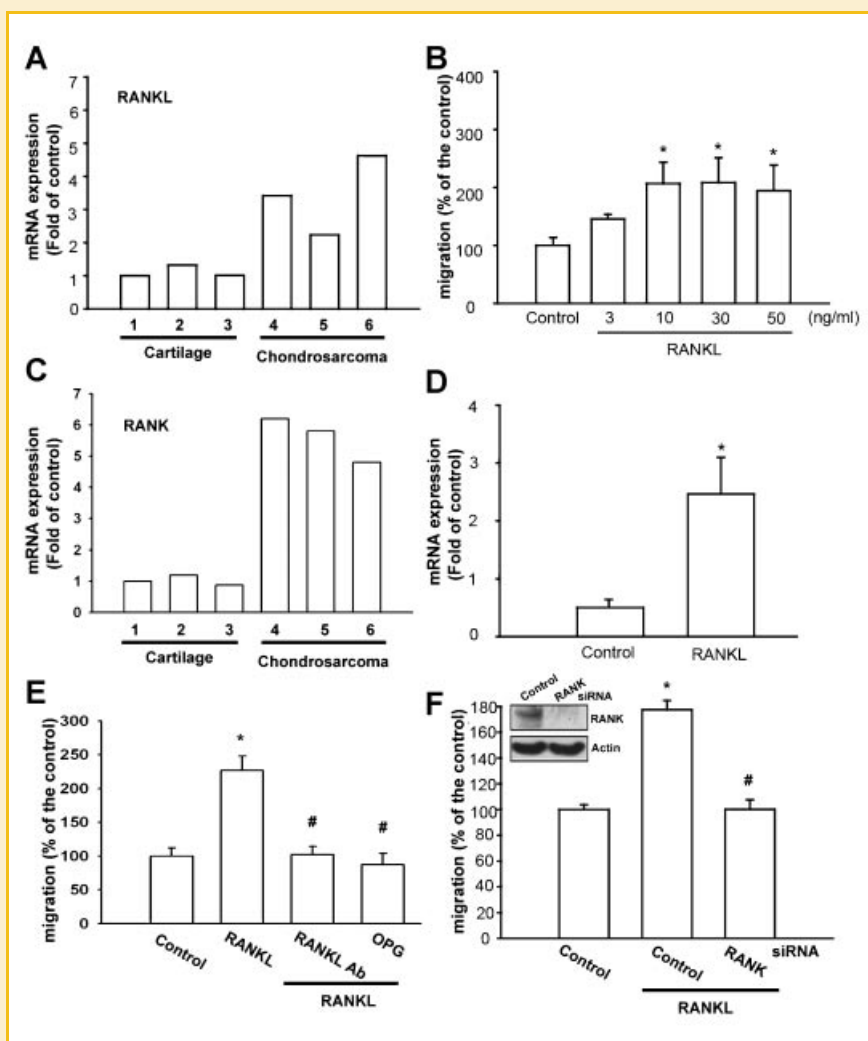

Fig. 1. RANKL/RANK axis–directed migration of human chondrosarcoma cells. A: Total RNA were extracted from normal cartilage (lines 1–3) or from chondrosarcoma patients (lines 4–6) and was subjected to qPCR analysis for RANKL. B: JJ012 cells were incubated with various concentrations of RANKL, and in vitro migration activity was measured with the Transwell after 24 h. C: Total RNA were extracted from normal cartilage (lines 1–3) or from chondrosarcoma patients (lines 4–6) and subjected to qPCR analysis for RANK. D: JJ012 cells were incubated with RANKL for 24 h, and the mRNA expression of RANK was examined by qPCR. Cells were pretreated for 30 min with RANK–Fc, OPG (E) or transfected with RANK siRNA (F) for 24 h. Then they were followed by stimulation with RANKL, and in vitro migration was measured with the Transwell after 24 h. Results are expressed as the mean ± SE. $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared with RANKL–treated group.

premixed for 20 min and then applied to cells. After 24 h transfection, the cells were then incubated with the indicated agents. After a further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

STATISTICS

The values given are means ± SEM. The significance of difference between the experimental groups and controls was assessed by Student's t-test. The difference was significant if the P value was <0.05.
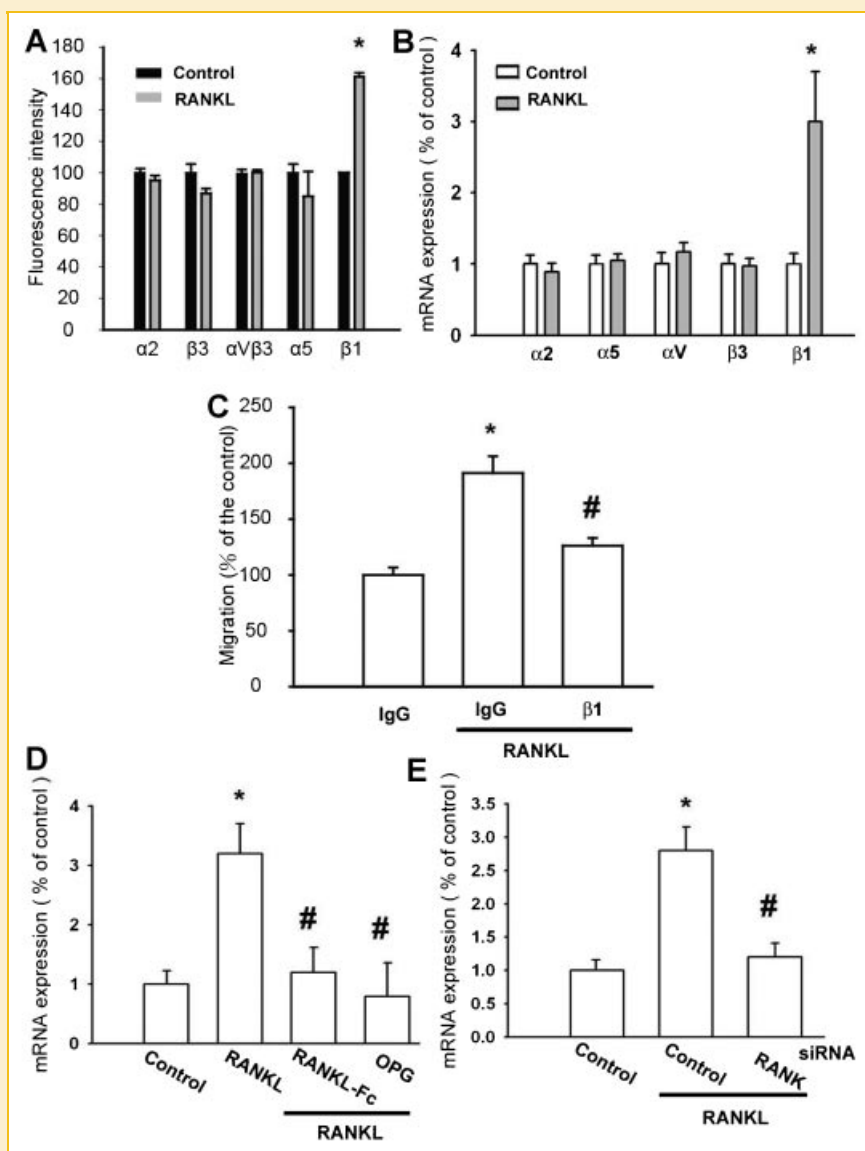

Fig. 2. RANKL–directed migration activity of human chondrosarcoma cells involves up–regulation of β1 integrin. A: JJ012 cells were incubated with RANKL (30 ng/ml), and the cell surface expression of α2, β3, α5, β1, and αvβ3 integrin was determined using flow cytometry. B: JJ012 cells were incubated with RANKL (30 ng/ml), and mRNA expression of α2, β3, α5, αv, and β1 integrin was determined using qPCR. C: Cells were pretreated with β1 monoclonal antibody for 30 min followed by stimulation with RANKL (30 ng/ml). The in vitro migration activity measured after 24 h. D,E: Cells were pretreated with RANKL–Fc and OPG for 30 min or transfected with RANK siRNA for 24 h followed by stimulation with RANKL (30 ng/ml) and mRNA expression of β1 integrin was determined using qPCR. Results are expressed as the mean ± SE. *P < 0.05 compared with control; #P < 0.05 compared with RANKL-treated group.

RESULTS

RANKL AND RANK INTERACTION DIRECT MIGRATION OF CHONDROSARCOMA CELLS

RANKL has been suggested to stimulate directional migration and invasion of human cancer cells [Armstrong et al., 2008; Heymann et al., 2008]. In this study, we first examined whether RANKL expresses in human chondrosarcoma cells. RANKL mRNA expression levels in chondrosarcoma patients (Fig. 1A, lines 4–6) were significantly higher than those in normal cartilage (Fig. 1A, lines 1–3). RANKL-triggered migration in chondrosarcoma cells was examined by using the Transwell assay with correction of RANKL-induced proliferation effects on human chondrosarcoma cells [Fong et al., 2008]. RANKL directed human chondrosarcoma cell (JJ012 cell) migration (Fig. 1B). Because interaction of RANKL with its specific receptor RANK on the surface of cancer cells has been suggested to induce cancer invasion [Chen et al., 2006; Heymann et al., 2008], we also examined RANK expression of human chondrosarcoma patients by qPCR. RANK mRNA expression levels in chondrosarcoma patients (Fig. 1C, lines 4–6) were

significantly higher than those in normal cartilage (Fig. 1C, lines 1–3). Stimulation of cells with RANKL increased the mRNA expression of RANK (Fig. 1D). Pretreatment of cells with RANKL-Fc or OPG (decoy receptor for RANKL) [Van Campenhout and Golledge, 2009] reduced RANKL-induced cell migration (Fig. 1E). However, transfected of cells with RANK siRNA reduced RANK protein expression (Fig. 1F) and antagonized RANKL-increased migration activity (Fig. 1F). Therefore, RANKL and RANK interaction plays a very important role in chondrosarcoma cells migration.

RANKL-DIRECTED CHONDROSARCOMA CELLS MIGRATION INVOLVES β1 INTEGRIN UP-REGULATION

Previous studies have shown significant expression of integrins in human chondrosarcoma cells [Yeh et al., 2008; Lai et al., 2009]. Therefore, we hypothesized that integrins may be involved in RANKL-directed chondrosarcoma cells migration. Flow cytometry analysis showed that RANKL-induced the cell surface expression of β1 but not α2, α5, β3, and αvβ3 integrin (Fig. 2A). In addition, RANKL also increased the mRNA expression of β1 but not α2, α5, and β3 integrin (Fig. 2B). However, RANKL slightly increased the mRNA expression
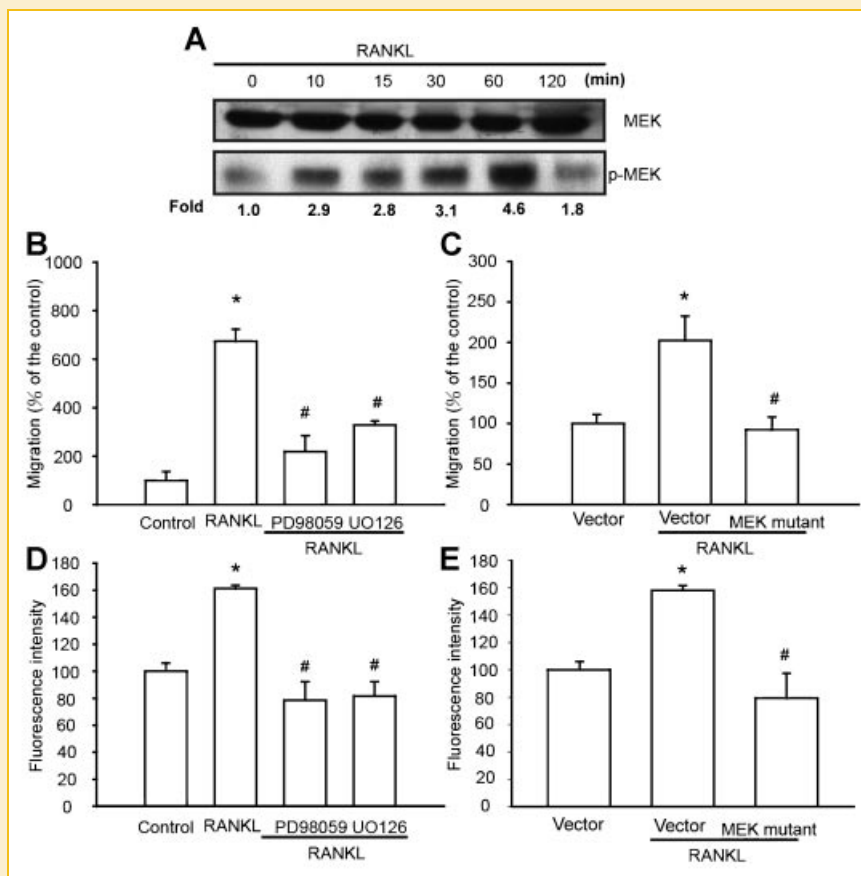

Fig. 3. MEK is involved in RANKL–induced migration and integrin up–regulation in human chondrosarcoma cells. A: JJ012 cells were incubated with RANKL (30 ng/ml) for indicated time intervals, and p–MEK was examined by Western blot analysis. B,C: JJ012 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 h followed by stimulation with RANKL (30 ng/ml), and in vitro migration was measured with the Transwell after 24 h. D,E: JJ012 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 h followed by stimulation with RANKL, and the cell surface β1 integrin was measured by using flow cytometry. Results are expressed as the mean ± SE. *$P < 0.05$ compared with control; #$P < 0.05$ compared with RANKL–treated group.

of αv integrin (Fig. 2B). Pretreatment of cells for 30 min with anti-β1 monoclonal antibody (mAb) (3 μg/ml) markedly inhibited the RANKL-induced cancer migration (Fig. 2C). Furthermore, RANKL increased β1 integrin expression was abolished by RANKL-Fc and OPG or RANK siRNA (Fig. 2D,E). We suggest RANKL and RANK interaction involves β1 integrin regulation.

MEK AND ERK SIGNALING PATHWAYS ARE INVOLVED IN THE RANKL-INDUCED INTEGRIN UP-REGULATION AND MIGRATION OF HUMAN CHONDROSARCOMA CELLS

MEK/ERK signaling pathway can be activated by a variety of growth factors, such as insulin and nerve growth factors [Chen et al., 2007; Knauf and Fagin, 2009]. We then examined whether RANKL stimulation enhances the activation of MEK/ERK pathway. After stimulation of JJ012 cells with RANKL led to a significant increase of phosphorylation of MEK1/2 by Western blot analysis (Fig. 3A), RANKL-induced the migration of JJ012 cells were greatly reduced by treatment with MEK inhibitors (PD98059 and U0126) (Fig. 3B). In addition, transfection of cells with MEK1 mutant reduced RANKL-induced cell migration (Fig. 3C). The MEK inhibitors (PD98059 and U0126) and mutant also inhibited the RANKL-increased β1 integrin up-regulation (Fig. 3D,E).

Furthermore, we determined ERK phosphorylation in response to RANKL in order to examine the crucial role of MEK/ERK in cancer migration and integrin up-regulation after treatment of JJ012 cells with RANKL resulted in significant phosphorylation of ERK (Fig. 4A). In addition, RANKL also increased ERK activity by determining phosphorylation of one of its substrates, ELK. Transfection with ERK2 mutant antagonized the RANKL-induced migration activity and β1 integrin expression in JJ012 cells (Fig. 4B,C). However, transfection of cells with MEK or ERK mutant did not affect the basal migration activity (Supplementary Fig. S1). Taken together, these results indicate that the MEK and ERK pathways are involved in RANKL-induced migration activity and β1 up-regulation in human chondrosarcoma cells.

NF-κB SIGNALING PATHWAY IS INVOLVED IN THE RANKL-INDUCED INTEGRIN UP-REGULATION AND MIGRATION ACTIVITY

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [Fong et al., 2008; Yeh et al., 2008]. To examine whether NF-κB activation is involved in RANKL-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Figure 5A shows that JJ012 cells pretreated with PDTC (10 μM) inhibited RANKL-induced chondrosarcoma cell migration. Furthermore, JJ012 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also inhibited RANKL-induced cancer cell migration (Fig. 5A). In addition, treatment of cells with PDTC or TPCK also antagonized RANKL-induced the expression of β1 integrin (Fig. 5B). To directly determine NF-κB activation after RANKL treatment, JJ012 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Figure 5C, RANKL treatment of JJ012 cells for 24 h increased in κB-luciferase activity dose-dependently. These results indicated that NF-κB activation is
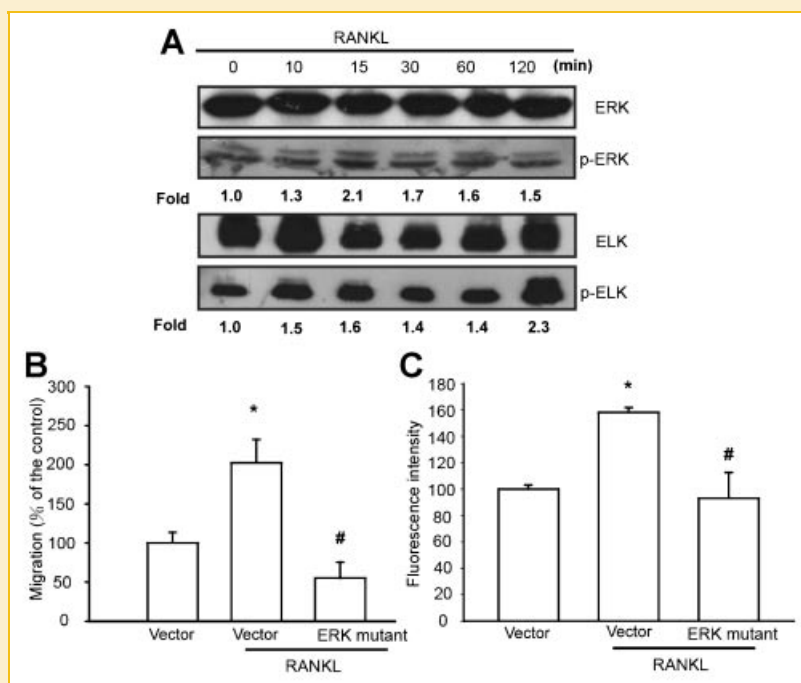

Fig. 4. ERK is involved in RANKL-induced migration and integrin up-regulation in human chondrosarcoma cells. A: JJ012 cells were incubated with RANKL (30 ng/ml) for indicated time intervals, and ERK phosphorylation and kinase activity was examined by Western blot analysis and kinase assay kit. B: JJ012 cells were transfected with dominant negative (DN) mutant of ERK2 for 24 h followed by stimulation with RANKL (30 ng/ml), and in vitro migration was measured with the Transwell after 24 h. C: JJ012 cells were transfected with dominant negative (DN) mutant of ERK2 for 24 h followed by stimulation with RANKL (30 ng/ml), and the cell surface β1 integrin was measured using flow cytometry. Results are expressed as the mean ± SE. *P < 0.05 compared with control; #P < 0.05 compared with RANKL-treated group.
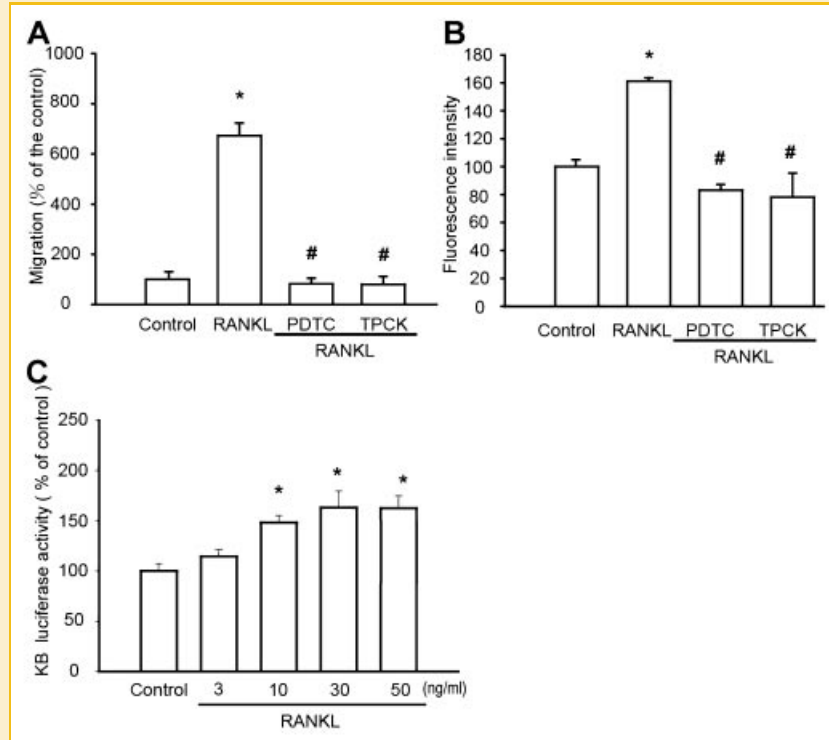
Fig. 5. RANKL induces cells migration and integrin up-regulation through NF-κB. A: JJ012 cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with RANKL (30 ng/ml), and in vitro migration was measured with the Transwell after 24 h. B: JJ012 cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with RANKL (30 ng/ml) for 24 h, and the cell surface β1 integrin was measured by flow cytometry. C: JJ012 cells were incubated with RANKL (30 ng/ml) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± SE. $^*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with RANKL-treated group.

important for RANKL-induced cancer cell migration and the expression of β1 integrin.

We further examined the upstream molecules involved in RANKL-induced NF-κB activation. Stimulation of JJ012 cells with RANKL induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6A). Meanwhile, cells transfected with IKKα or IKKβ mutant markedly inhibited the RANKL-induced cancer cells migration and integrin up-regulation (Fig. 6B,C). However, IKKα or IKKβ mutant did not affect the basal migration activity and integrin expression in chondrosarcoma cells (Supplementary Fig. S1). These data suggest that IKKα/β activation is involved in RANKL-induced the migration activity of human chondrosarcoma cells. Treatment of RANKL in chondrosarcoma cells 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; Viatour et al., 2005], and the antibody specific against phosphorylated p65 Ser[536] was used to examine p65 phosphorylation. Treatment of JJ012 cells with RANKL for various time intervals resulted in p65 Ser[536] phosphorylation (Fig. 6A). Pretreatment of cells with PD98059 or U0126 inhibited RANKL-induced p65 Ser[536] phosphorylation (Fig. 6D). Therefore, NF-κB may function as a downstream signaling molecule of MEK/ERK in the RANKL signaling pathway. In addition, PD98059, U0126, PDTC, or TPCK antagonized the RANKL-induced κB-luciferase activity (Fig. 6E). Co-transfection of cells with MEK, ERK, IKKα, or

IKKβ mutant also reduced RANKL-increased κB-luciferase activity (Fig. 6F). In addition, these inhibitors and mutants did not affect the basal κB-luciferase activity (Supplementary Fig. S1). Taken together, these data suggest that activation of MEK/ERK is required for RANKL-induced NF-κB activation in chondrosarcoma cells.

DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which cause dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to absence of an effective adjuvant therapy [Fong et al., 2007]. The metastatic potential for conventional chondrosarcomas correlates well with the histologic grade of the tumor. But due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, ~15% of patients dying from metastatic disease do so >5 years after initial diagnosis [Fong et al., 2007]. Therefore, it is important to develop effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that RANKL and its RANK receptor would help to direct the metastasis of chondrosarcoma cells. We found that RANKL increased the migration of chondrosarcoma cells. One of the mechanisms underlying RANKL-directed migration was transcriptional up-regulation of β1 integrin and activation of
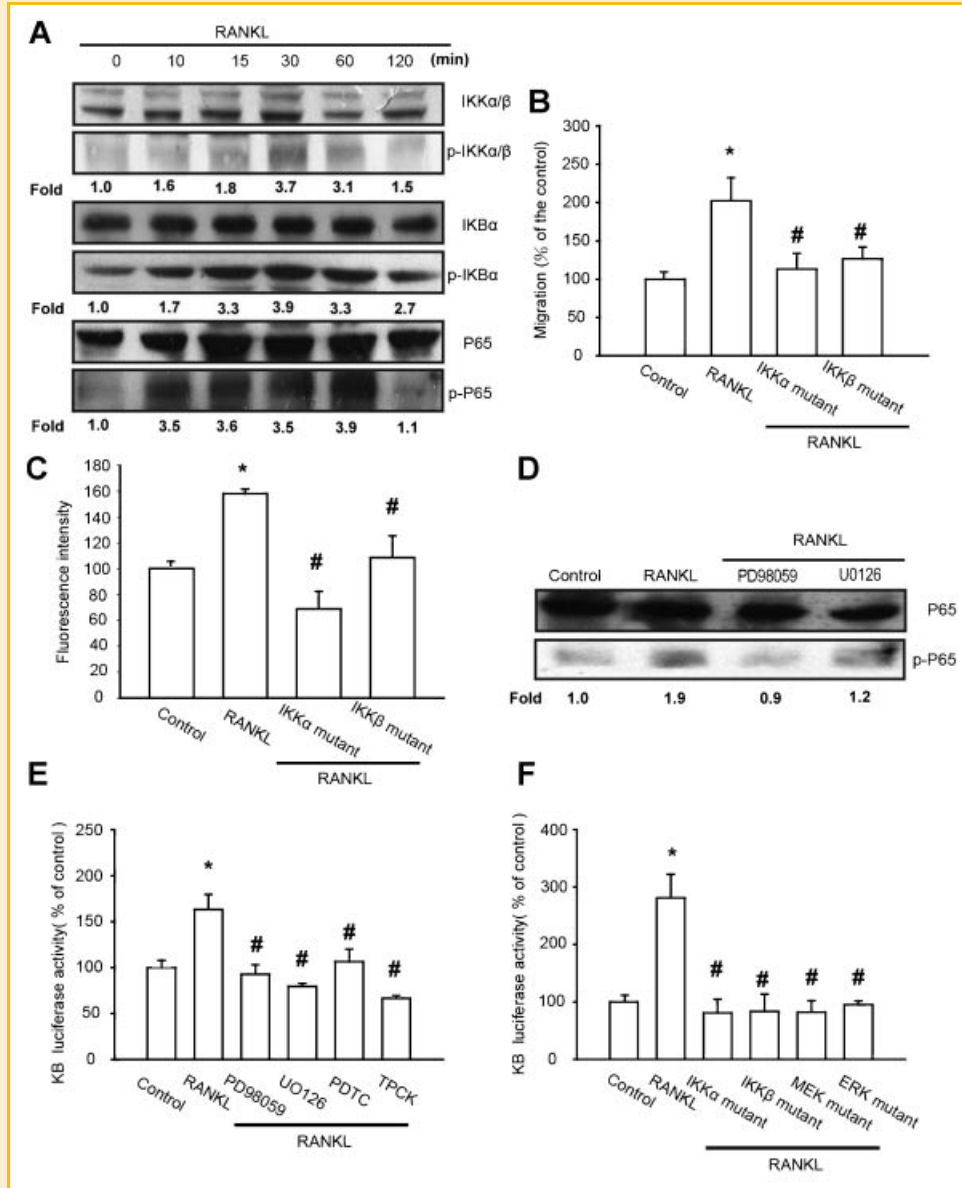
Fig. 6. MEK/ERK pathway is involved in RANKL–mediated NF-κB activation and integrin expression. A: JJ012 cells were incubated with RANKL (30 ng/ml) for indicated time intervals, and IKK, IκBα, and p65 phosphorylation was examined by Western blot analysis. B: JJ012 cells were transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 h followed by stimulation with RANKL (30 ng/ml), and in vitro migration was measured with the Transwell after 24 h. C: JJ012 cells were transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 h followed by stimulation with RANKL (30 ng/ml), and the cell surface β1 integrin was measured by using flow cytometry. D: Cells were pretreated with PD98059 or U0126 for 30 min. Then they were followed by stimulation with RANKL (30 ng/ml) for 60 min, and p65 phosphorylation was examined by Western blot analysis. E: Cells were pretreated with U0126, PD98059, PDTC, and TPCK for 30 min or transfected with mutant of MEK, ERK, IKKα, and IKKβ (F) before exposure to RANKL (30 ng/ml). NF-κB luciferase activity was measured, and the results were normalized to the β–galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ compared with control; $^{#}P < 0.05$ compared with RANKL–treated group.

RANK, MEK, ERK, and NF-κB pathways. In addition, RANKL-Fc, OPG, β1 integrin mAb, PD98059, U0126, PDTC, and TPCK reduced RANKL-mediated cell migration in other chondrosarcoma, SW1353 cell line (data not shown). On the other hand, RANKL-Fc, OPG, PD98059, U0126, PDTC, and TPCK also abolished RANKL-increased β1 integrin expression in SW1353 cells (data not shown). Therefore, the same signaling pathways of migration are involved in all chondrosarcoma cell lines. As the primary chondrosarcoma migration is also through this signaling pathway, it needs further study.

RANKL and its receptor, RANK, play a key role in osteoclasto-genesis and tumor metastasis [Kearns et al., 2008]. The expression of the RANKL and RANK receptor in human chondrosarcoma cells are mostly unknown. In this study, we found that human chondro-sarcoma cells expressed the mRNA of RANKL. By using qPCR analysis, we also found that primary chondrosarcoma cells express

RANK receptor. In addition, the expression of mRNA levels of RANK in chondrosarcoma patients was significantly higher than those in normal cartilage. On the other hand, JJ012 cells stimulated with RANKL increased RANK mRNA expression. Our data provided the evidence that the expression of RANKL and RANK are associated with a metastatic phenotype of chondrosarcoma cells. Moreover, RANKL-Fc, OPG, or RANK siRNA reduced RANKL-mediated cell migration. Therefore, RANKL and RANK interaction is mediated migration activity in human chondrosarcoma cells.

A variety of growth factors stimulate the expression of integrin via signal-transduction pathways that converge to activate NF-κB complex of transcription factors [Fong et al., 2009; Huang et al., 2009]. The MEK/ERK pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [Kapur et al., 2003]. We found RANKL-enhanced MEK phosphorylation in human chondrosarcoma cells. Pretreatment of cells with MEK inhibitor U0126 or PD98059 antagonized the increase of migration and integrin expression by RANKL stimulation. This was further confirmed by the result that the dominant-negative mutant of MEK1 inhibited the enhancement of migration by RANKL. Moreover, we also found that RANKL activated ERK phosphorylation and kinase activity, while ERK mutant inhibited RANKL-mediated migration activity and integrin up-regulation. Our data indicates that MEK/ERK might play an important role in the expression of integrin and migration of human chondrosarcoma cells.

Many NF-κB activation pathways have been suggested, and all of them rely on sequentially activated kinase cascades. The classical pathway is triggered by various pro-inflammatory cytokines such as IL-1β and TNF-α. These extracellular signals activate the IKK complex which phosphorylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription. Besides the phosphorylation and degradation of the IκB signal pathway, an IκB-independent pathway such as p65 phosphorylation for optimal NF-κB activation has been defined [Viatour et al., 2005]. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser^{536} through IKKs, resulting in increased transcriptional activity of p65 [Sakurai et al., 1999]. The results of this study showed that RANKL increased the phosphorylation of IKKα/β, IκBα and p65. On the other hand, PD98059, U0126, PDTC, and TPCK reduced RANKL-mediated NF-κB promoter activity. Our data indicated that MEK/ERK and NF-κB pathway might play important roles in the expression of β1 integrin and migration of human chondrosarcoma cells. In this study, we also found that MEK/ERK inhibitors (PD98059 and U0126) reduced RANKL-induced p65 phosphorylation. Therefore, NF-κB may function as a downstream signaling molecule of MEK/ERK in the RANKL signaling pathway. As for the other molecules that are involved in RANKL-mediated cell migration, they need further examination.

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 RANKL increases the activity of β1 integrin via the RANK, MEK, ERK, IKKαβ, and NF-κB-dependent pathway and to enhance migration of human chondrosarcoma cells. Furthermore, the discovery of RANKL/RANK-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

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