TNF-*α* increases αvβ3 integrin expression and migration in human chondrosarcoma cells

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Abstract

Chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and cause distant metastasis. Chondrosarcoma shows a predilection for metastasis to the lungs. Tumor necrosis factor (TNF)-*α* is a key cytokine involved in inflammation, immunity, cellular homeostasis and tumor progression. Integrins are the major adhesive molecules in mammalian cells and have been associated with metastasis of cancer cells. However, the effects of TNF-*α* in migration and integrin expression in chondrosarcoma cells are largely unknown. In this study, we found that TNF-*α* increased the migration and the expression of αvβ3 integrin in human chondrosarcoma cells. Activations of MAPK kinase (MEK), extracellular signal-regulating kinase (ERK) and nuclear factor-κB (NF-κB) pathways after TNF-*α* treatment were demonstrated, and TNF-*α*-induced expression of integrin and migration activity was inhibited by the specific inhibitor and mutant of MEK, ERK and NF-κB cascades. Taken together, our results indicated that TNF-*α* enhances the migration of chondrosarcoma cells by increasing αvβ3 integrin expression through the MEK/ERK/NF-κB signal transduction pathway.

Running title: TNF-*α* induces the migration of chondrosarcoma

Key words: TNF-*α*; Migration; Chondrosarcoma; ERK; NF-κB

Introduction

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).

Tumor necrosis factor (TNF)-α is a key cytokine involved in inflammation, immunity, cellular homeostasis and tumor progression (Balkwill, 2009). TNF-α is synthesised as a transmembrane protein with a molecular mass of 26 kDa and the pro-peptide is secreted as a soluble 17-kDa molecule on cleavage by TNF-α-converting enzyme (Cross et al., 2006). Accumulating evidence has shown that TNF-α is a key mediator of inflammation and cancer (Balkwill, 2009). Constitutive production of TNF-α from the tumor microenvironment is a characteristic of many malignant tumors (Bradley, 2008). TNF-α directly increases proliferation and survival in cancer cells (Balkwill, 2006) and it can also exert its effects indirectly through increases endothelial cells and other inflammatory cells proliferation and activation presented at the tumor microenvironment (Balkwill, 2009; Li et al., 2009). NF-κB is activated and leads to the expression of a variety of inflammation-related genes (Wang et al., 2009). Transient activation of NF-κB in response to stimulation by cytokines induces the inflammatory response; however, sustained activation of NF-κB has been associated with several aspects of oncogenesis, such as promoting cancer cell proliferation, preventing apoptosis in drug resistance and increasing tumor angiogenesis and metastasis (Keibel et al., 2009).

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 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 19 α and 8 β 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 (Heyder et al., 2005; Seales et al., 2005; Takenaka et al., 2000).

Previous studies have shown that TNF-*α* modulates cell migration and invasion in human gastrointestinal, melanoma and hepatocarcinoma cells (Katerinaki et al., 2003; Nejjari et al., 1999; Ziprin et al., 2003). However, the effect of TNF-*α* on integrin expression and migration activity in human chondrosarcoma cells is mostly unknown. In this study, we explored whether TNF-*α* 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 cell migration by TNF-*α*.

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, p-IκBα, p65, α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody specific for αvβ3 integrin was purchased from Chemicon (Temecula, CA). U0126, PD98059, TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for phosphor-IKKα/β ($Ser^{180/181}$) and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA, USA). The recombinant human TNF-*α* was purchased from PeproTech (Rocky Hill, NJ, USA). 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, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The human chondrosarcoma cell line (JJ012) was kindly provided by the laboratory of Dr. Sean P. Scully (University of Miami School of Medicine, Miami, FL, USA). The human chondrosarcoma cell line (SW1353) was obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium 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, USA; 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). 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 TNF-*α* 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 TNF-α 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 hr 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, USA) 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 hr at room temperature and then probed with rabbit anti-human antibodies against IκBα, p-IκB or IKKα/β (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak,

Rochester, NY, USA).

Quantitative real-time PCR

Total RNA was extracted from chondrosarcomas using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two μg of total RNA was reverse transcribed into cDNA 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 ng 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, CA). qPCR assays were carried out in triplicate on a 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).

Immunofluorocytochemistry

Cells were cultured in 12-mm coverslips. After treatment with TNF-*α*, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% non-fat milk in PBS containing 0.5% Triton X-100 was added to the cells. The cells were then incubated with rabbit anti-p65 (1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St. Louis, MO, USA) for 1 h. The FITC was detected using a Zeiss fluorescence microscope.

Synthesis of NF-κB decoy oligonucleotide (ODN)

We used a phosphorothioate double-stranded decoy ODN carrying the NF-κB/Rel-consensus sequence 5’-CCTTGAA

GGGATTTCCCTCC-3'/3'-GGAACTTCCCTAAAGGGAGG-5'. The mutated (scrambled) form 5'-TTGCCGTACCTGACTTAGCC-3'/3'-AACGGCATGGACTGAATCGG-5' was used as a control. ODN (5 μM) was mixed with Lipofectamine 2000 (10μg/ml) for 25 min at room temperature and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments (Tang et al., 2008).

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, USA). DNA and LF2000 were premixed for 20 min and then applied to cells. After 24 hr transfection, the cells were then incubated with the indicated agents. After a further 24 hr incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI, USA) 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 ± S.E.M. The significance of differences between the experimental groups and controls was assessed by Student's t test. p values <0.05 were considered significant.

Results

TNF-α-directed chondrosarcoma cells migration involves αvβ3 integrin up-regulation

TNF-*α* has been suggested to stimulate directional migration and invasion of human gastrointestinal, melanoma and hepatocarcinoma cells (Balkwill, 2009). TNF-*α*-triggered migration in chondrosarcoma cells was examined using the Transwell assay with correction of TNF-*α*-induced proliferation effects on human chondrosarcoma cells (Fong et al., 2008). TNF-*α* directed human chondrosarcoma cell (JJ012 cell) migration (Fig. 1A). In addition, TNF-*α* also dose-dependently directed other human chondrosarcoma cell migration (SW1353 cells) (Fig. 1B). Previous studies have shown significant expression of αvβ3 integrin in human chondrosarcoma cells (Chen et al., 2009; Lai et al., 2009). We therefore, hypothesized that αvβ3 integrin may be involved in TNF-*α*-directed chondrosarcoma cell migration. Flow cytometry analysis showed that TNF-*α* induced the cell surface expression of αvβ3 integrin dose-dependently (Fig. 2A). To confirm this finding, expression of mRNA for the integrins in response to TNF-*α* was analyzed by Western blot and qPCR. Treatment of JJ012 cells with TNF-*α* induced the protein and mRNA expression of αv and β3 integrin (Fig. 2B&C). Pretreatment of cells for 30 min with anti-αvβ3 monoclonal antibody (mAb) (10 μg/ml) markedly inhibited the TNF-*α*-induced cell migration (Fig. 2C). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind αvβ3 at high affinity and block its function effectively at low concentrations (Brooks et al., 1996). Treatment of cells with cyclic RGD, but not cyclic RAD, inhibited the TNF-*α*-induced migration of chondrosarcoma (Fig. 2C). These data suggest that TNF-*α*-induced cancer migration may occur via activation of αvβ3 integrin receptor.

MEK and ERK signaling pathways are involved in the αvβ3-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 TNF-α stimulation enhances the activation of the MEK/ERK pathway. Stimulation of JJ012 cells with TNF-α led to a significant increase in phosphorylation of MEK1/2, as shown by Western blot analysis (Fig. 3A), and TNF-α-induced migration of JJ012 cells was greatly reduced by treatment with MEK inhibitors (PD98059 and U0126) (Fig. 3B). In addition, transfection of cells with MEK1 mutant reduced TNF-α-induced cell migration (Fig. 3D). The MEK inhibitors (PD98059 and U0126) and mutant also inhibited the TNF-α-increased $\alpha v\beta 3$ integrin up-regulation (Fig. 3C&E). Furthermore, we determined ERK phosphorylation in response to TNF-α in order to examine the crucial role of MEK/ERK in cancer migration, and integrin up-regulation after treatment of JJ012 cells with TNF-α resulted in significant phosphorylation of ERK (Fig. 4A). Transfection with ERK2 mutant antagonized the TNF-α-induced migration activity and $\alpha v\beta 3$ integrin expression in JJ012 cells (Fig. 4B&C). Taken together, these results indicate that the MEK and ERK pathways are involved in TNF-α-induced migration activity and $\alpha v\beta 3$ up-regulation in human chondrosarcoma cells.

NF-κB signaling pathway is involved in TNF-α-induced integrin up-regulation and migration activity

As mentioned above, 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 TNF-α-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 5A shows that JJ012 cells pretreated with PDTC (10 μM) inhibited TNF-α-induced chondrosarcoma cell migration. Furthermore, JJ012 cells pretreated with TPCK (3μM), an IκB protease inhibitor, also inhibited TNF-α-induced cancer cell migration (Fig. 5A). Furthermore, the increase of migration activity by TNF-α was antagonized by cis element decoy agonist NF-κB-binding site (decoy NF-κB ODN), but not by scrambled decoy ODN (Fig. 5B).

Transfection of cells with NF-κB ODN, but not scrambled decoy ODN, reduced TNF-*α*-induced protein and mRNA expression of αvβ3 integrin (Supplementary Figure S1). In addition, treatment of cells with PDTC or TPCK also antagonized TNF-*α*-induced expression of αvβ3 integrin (Fig. 5C). To directly determine NF-κB activation after TNF-*α* treatment, JJ012 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig 5D, TNF-*α* treatment of JJ012 cells for 24 hr increased κB-luciferase activity dose-dependently. These results indicated that NF-κB activation is important for TNF-*α*-induced cancer cell migration and the expression αvβ3 integrin.

We further examined the upstream molecules involved in TNF-*α*-induced NF-κB activation. Stimulation of JJ012 cells with TNF-*α* induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6A). Treatment of TNF-*α* in chondrosarcoma cells also caused IκBα phosphorylation in a time-dependent manner (Fig. 6A). In addition, treatment of JJ012 cells with TNF-*α* for various time intervals resulted in p65 phosphorylation (Fig. 6A). Pretreatment of cells with PD98059 or U0126 inhibited TNF-*α*-induced IKK and p65 phosphorylation or p65 translocation into nucleus (Fig. 6B&C). Therefore, NF-κB may function as a downstream signaling molecule of MEK/ERK in the TNF-*α* signaling pathway. In addition, PD98059 or U0126 antagonized the TNF-*α*-induced κB-luciferase activity (Fig. 6D). Co-transfection of cells with MEK, ERK, IKKα or IKKβ mutant also reduced TNF-*α*-increased κB-luciferase activity (Fig. 6D). Taken together, these data suggest that activation of MEK/ERK is required for TNF-*α*-induced NF-κB activation in chondrosarcoma cells.

Discussion

Integrins link the ECM 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 (Shattil et al.). Here we found that TNF-*α* increased αvβ3 integrin expression by using flow cytometry analysis, which plays an important role during tumor metastasis (Beer and Schwaiger, 2008). Furthermore, TNF-*α* also increased the mRNA levels of αv and β3 integrins. It has been reported that TNF-*α* increased expression of α5β1 integrin (Sun et al.). Furthermore, αvβ3 and αvβ5 integrin also mediated TNF-α-induced cell adhesion (Bieler et al., 2007). In this study, we examined the effects of αvβ3 integrin in TNF-*α*-mediated cell migration. Whether or not other integrins are involved in TNF-*α*-increased chondrosarcoma migration remains unclear. It has been reported that TNF-*α* increased neutrophil adhesion through activation of α5β1 integrin without increasing their protein expression (Sun et al.). Here we found that TNF-α increased αv and β3 cell surface, mRNA and protein expression. These results indicate that increasing cell surface integrins is sufficient to induce cell adhesion and migration ability. In the present study, we used cyclic RGD to determine the role of αvβ3 integrin and found that it inhibited TNF-*α*-induced cancer migration, indicating the possible involvement of αvβ3 integrin in TNF-*α*-induced migration in chondrosarcoma cells. This was further confirmed by the result that the αvβ3 integrin antibody inhibited the enhancement of migration activity by TNF-*α*, indicating the involvement of αvβ3 integrin in TNF-*α*-mediated induction of chemomigration.

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 activator of the NF-κB signaling pathway in human cancer cells (Kapur et al., 2003). We found TNF-*α*-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 TNF-*α* stimulation. This was further confirmed by our finding which demonstrated that the dominant-negative mutant of MEK1 inhibited the enhancement of migration by TNF-*α*. Moreover, we also found that TNF-*α* activated ERK phosphorylation, while

ERK mutant inhibited TNF-*α*-mediated migration activity and integrin up-regulation. Our data indicate 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 and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription. 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 show that TNF-*α* increased the phosphorylation of IKKα/β, IκBα and p65. In addition, pretreatment of cells with PD98059 and U0126 reduced TNF-*α*-increased IKK and p65 phosphorylation or p65 translocation into the nucleus. However, PD98059 and U0126 reduced TNF-*α*-mediated NF-κB promoter activity. Our data indicate that MEK/ERK and NF-κB pathway might play important roles in the expression of αvβ3 integrin and migration of human chondrosarcoma cells. It has been reported that αv integrin and β3 integrin promoter region contain NF-κB binding site, respectively (Li et al., 2006; Sharma et al., 1995). Therefore, the same signaling pathways (MEK/ERK and p65) activate NF-κB binding on αv integrin and β3 integrin promoter and contribute to cell migration.

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which have seen 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 who die 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 TNF-α would help to direct the metastasis of chondrosarcoma cells. In addition, metastasis is a complex process, and migration is a few steps removed from metastasis. Here we used migration assay to examine the cell migration activity. However, further research is needed on the *in vivo* metastasis effect of TNF-α. We found that TNF-α increased the migration of chondrosarcoma cells. However, TNF-α induces only a 2-fold increases in migration of chondrsarcoma cells. In our previous study, we found a maximum 3-fold increase in migration of chondrosarcoma cells induced by another chemokine (CCL5) (Tang et al.). Therefore, the maximum increase in migration of chondrosarcoma cells is ~2- to 3-fold. One of the mechanisms underlying TNF-α-directed migration was up-regulation of αvβ3 integrin and activation of MEK, ERK and NF-κB pathways. In addition, αvβ3 integrin mAb, PD98059, U0126, PDTC and TPCK reduced TNF-α-mediated cell migration in SW1353 cells (Supplementary Figure S2). Stimulation of SW1353 cells with TNF-α increased cell surface αvβ3 integrin dose-dependently. Furthermore, PD98059, U0126, PDTC and TPCK also abolished TNF-α-increased αvβ3 integrin expression in SW1353 cells (Supplementary Figure S2). Therefore, the same signaling pathways are involved in all chondrosarcoma cells. Our findings demonstrated that TNF-α increases the activity of αvβ3 integrin via the MEK, ERK, IKKα/β and NF-κB-dependent pathway and enhances migration of human chondrosarcoma cells (Fig. 6E). This signaling pathway also affects TNF-α-increased migration in breast cancer cells (Supplementary Figure S3). Therefore, this pathway may be a common pathway in TNF-α-mediated cell migration. Whether this pathway is involved in the migration of other types of cancer cells warrants further investigation. Furthermore, the discovery of TNF-α-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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Figure legends

Fig. 1 TNF-*α*-directed migration of human chondrosarcoma cells

JJ012 (A) or SW1353 (B) cells were incubated with TNF-*α* for 24 hr, and *in vitro* migration was measured by Transwell after 24 hr. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control.

Fig. 2 TNF-*α*-directed migration activity of human chondrosarcoma cells involves up-regulation of αvβ3 integrin.

(A) JJ012 cells were incubated with TNF-*α*, and the cell surface expression of αvβ3 integrin was determined using flow cytometry. (B&C) JJ012 cells were incubated with TNF-*α*, and protein and mRNA expression of αv and β3 integrin was determined using Western blot and qPCR. (D) Cells were pretreated with αvβ3 monoclonal antibody, cyclic RGD (100 nM) or cyclic RAD (100 nM) for 30 min followed by stimulation with TNF-*α*. The *in vitro* migration activity was measured after 24 hr. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TNF-*α*-treated group.

Fig. 3 MEK is involved in TNF-*α*-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with TNF-*α* for indicated time intervals, and p-MEK was examined by Western blot analysis. (B&D) JJ012 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 hr followed by stimulation with TNF-*α*, and *in vitro* migration was measured by Transwell after 24 hr. (C&E) JJ012 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 hr followed by stimulation with TNF-*α*, and the cell surface αvβ3 integrin was measured using flow cytometry. Results are expressed as the mean ± S.E. *, $p < 0.05$

compared with control; #, $p < 0.05$ compared with TNF-α-treated group.

Fig. 4 ERK is involved in TNF-α-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with TNF-α for indicated time intervals, and ERK phophorylation and kinase activity were examined by Western blot analysis. (B) JJ012 cells were transfected with dominant negative (DN) mutant of ERK2 for 24 hr followed by stimulation with TNF-α, and *in vitro* migration was measured by the Transwell after 24 hr. (C) JJ012 cells were transfected with dominant negative (DN) mutant of ERK2 for 24 hr followed by stimulation with TNF-α, and the cell surface αvβ3 integrin was measured using flow cytometry. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TNF-α-treated group.

Fig. 5 TNF-α 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 TNF-α, and *in vitro* migration was measured by the Transwell after 24 hr. (B) JJ012 cells were transfected with NF-κB ODN or scrambled ODN for 24 h followed by stimulation with TNF-α for 24 h, and *in vitro* migration was measured by Transwell after 24 h. (C) JJ012 cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with TNF-α for 24 hr, and the cell surface αvβ3 integrin was measured by flow cytometry. (D) JJ012 cells were incubated with TNF-α for 24 hr. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TNF-α-treated group.

Fig. 6 MEK/ERK pathway is involved in TNF-α-mediated NF-κB activation and

integrin expression.

(A) JJ012 cells were incubated with TNF-α for indicated time intervals, and IKK, IκBα and p65 phophorylation was examined by Western blot analysis. JJ012 cells were pretreated with PD98059 and U0126 for 30 min. Then they were followed by stimulation with TNF-α for 120 min, and IKK or p65 phosphorylation (B) and p65 immunofluorescence staining (C) was examined. (D) Cells were pretreated with U0126 and PD98059 for 30 min or transfected with mutant of MEK, ERK, IKKα and IKKβ before exposure to TNF-α. NF-κB luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with TNF-α-treated group. (E) Schematic presentation of the signaling pathways involved in TNF-α-induced migration and αvβ3 integrin expression of chondrosarcoma cells. TNF-α activates MEK and ERK pathways, which in turn induces NF-κB activation, which lead to αvβ3 integrin expression and increases the migration of human chondrosarcoma cells.

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