BIOCHEMICAL PHARMACOLOGY 75 (2008) 1292–1301

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TGF-β1 increases motility and αvβ3 integrin up-regulation via PI3K, Akt and NF-κB-dependent pathway in human chondrosarcoma cells

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ARTICLE INFO

Article history:
Received 19 October 2007
Accepted 28 November 2007

Keywords:
TGF-β1
Chondrosarcoma
Integrin
Migration
NF-κB

ABSTRACT

Transforming growth factor-β1 (TGF-β1) plays an essential role in tumor progression and metastasis. Integrins are the major adhesive molecules in mammalian cells. Here we found that TGF-β1 increased the migration and cell surface expression of αvβ3 integrin in human chondrosarcoma cells (JJ012 cells). Phosphatidylinositol 3-kinase inhibitor (PI3K; Ly294002) or Akt inhibitor inhibited the TGF-β1-induced increase the migration of chondrosarcoma cells. TGF-β1 stimulation increased the phosphorylation of p85 subunit of PI3K, and serine 473 of Akt. In addition, treatment of JJ102 cells with NF-κB inhibitor (PDTC) or IκB protease inhibitor (TPCK) inhibited TGF-β1-induced cells migration and integrins expression. Treatment of JJ012 cells with TGF-β1-induced IκB kinase α/β (IKKα/β) phosphorylation, IκBα phosphorylation, p65 Ser^{536} phosphorylation, and κB-luciferase activity. The TGF-β1-mediated increases in IKKα/β phosphorylation and p65 Ser^{536} phosphorylation were inhibited by Ly294002 and Akt inhibitor. Cotransfection with p85 and Akt mutants also reduced the TGF-β1-induced κB-luciferase activity. Taken together, these results suggest that the TGF-β1 acts through PI3K/Akt, which in turn activates IKKα/β and NF-κB, resulting in the activations of αvβ3 integrins and contributing the migration of chondrosarcoma cells.

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Abbreviations: TGF-β1, transforming growth factor-β1; PI3K, phosphatidylinositol 3-kinase; IKKα/β, IκB kinase α/β; ECM, extracellular matrix; SDF-1, stromal cells-derived factor-1; NF-κB, nuclear factor-κB.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.11.017

1. 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 [1]. 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 [2] and therefore, it is important to explore a novel and adequate remedy.

Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and distant metastasis [3]. An approach 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 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 [4]. 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 allow cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen and vitronectin [5]. 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 [6]. Integrin has been heavily implicated in tumor development [7,8], was correlated to reduced patient survival in colon carcinoma and melanoma [9,10], and has been associated with breast cancer cell metastasis to bone [11]. In addition, αvβ3 integrin has been implicated in prostate cancer progression with effects on angiogenesis, survival and invasion [12,13]. *In vitro* studies have found that integrins facilitated prostate cancer cell adhesion and migration through several ECM substrates [12], and transendothelial migration [14]. Nuclear factor-κB (NF-κB) transcription factor represent a ubiquitously expressed protein family that modulates the expression of genes involved in diverse cellular functions, such as stress response, immune reactions, and metastasis [44]. NF-κB transcription is involved in αvβ3 expression in human endothelial cells [45]. It has been also reported that NF-κB plays a key in stromal cells-derived factor-1 (SDF-1)-induced chemomigration and β1 and β3 integrin up-regulation [46].

The transforming growth factor-β (TGF-β) family consists of three closely related isoforms (TGF-β1, -β2, and -β3) that are prototypes of the larger TGF-β superfamily [15,16]. TGF-β family members elicit a diverse range of cellular responses including cell proliferation, migration, fibrosis, inflammation, and wound repair [15,16]. The biological functions of TGF-β1 are of widespread importance in embryogenesis and in fibroproliferative disorders in adults, for example, liver cirrhosis, chronic glomerulonephritis, and atherosclerosis [17]. TGF-β1 knockout mice develop diffuse mononuclear cell infiltrates that prove lethal within a few weeks of birth [18]. Recent studies have suggested a fundamental role for TGF-β1 as a critical mediator of the metastasis activity of cancer cells [19]. Although the mechanisms underlying TGF-β1-mediated tumor invasion have been studied in some cancers [19], the role of TGF-β1 in the process of chondrosarcoma cells migration remains large unknown.

Previous studies have shown that TGF-β1 modulates cell migration and invasion in several cancer cells [19]. TGF-β1-mediated invasion may involve activation of integrins receptors [20,21]. However, the effect of TGF-β1 on integrins expression and migration activity in human chondrosarcoma cells is mostly unknown. We hypothesized that TGF-β1 might be capable of regulating chondrosarcoma cells migration and integrin expression. Here we found that TGF-β1 increased the migration and the expression of integrins of human chondrosarcoma cells. In addition, phosphatidylinositol 3-kinase (PI3K), Akt, IKKα/β and NF-κB signaling pathways may be involved in the increase of integrin expression and cells migration by TGF-β1.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-Akt, Akt, p85, phosphotyrosine residues (PY20), IKKα/β, p-IκBα, α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ly294002, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate)), 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 TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA). Rabbit polyclonal antibodies specific for αvβ3 integrin were purchased from Chemicon. A selective αvβ3 integrin antagonist cyclic RGD (cyclo-RGDfV) peptide and the cyclic RAD (cyclo-RADfV) peptide [22] were purchased from Peptides International (Louisville, KY). The p85 (Δp85; deletion of 35 amino acids from residues 479–513 of p85) and Akt (Akt K179A) dominant-negative mutants were gifts from Dr. R.H. Chen (Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan). 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 obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. 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) [23]. 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.

2.3. 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 Ly294002, Akt inhibitor, PDTC, TPCK or vehicle control (0.1% DMSO). Approximately 1×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing 10 ng/ml TGF-β1 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 migrating cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of the TGF-β1 treatment (corrected migrating cell number = counted migrating cell number/percentage of viable cells) [24,45].

2.4. 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 rinsed in PBS, the cells were incubated with rabbit anti-human antibody against αvβ3 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 (1:150; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences) [25]. The values given are crude fluorescence intensity recording by flow cytometry.

2.5. Western blot analysis

The cellular lysates were prepared as described previously [26]. 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-IκBα, IKKαβ or p-Akt (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

2.6. 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. JJ102 cells were grown to 80% confluent in 12-well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were then incubated with the indicated agents. After 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, Madison, WI) 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.

2.7. Statistics

The values given are means ± S.E.M. Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. In all cases, $p < 0.05$ was considered as significant.

3. Results

3.1. TGF-β1-directed chondrosarcoma cells migration involves αvβ3 integrin up-regulation

TGF-β1 has reported stimulates directional migration and invasion of human cancer cells [27]. The TGFβ-1 for chondrosarcoma cells migration was examined using the Transwell assay with correction of TGFβ-1-induced proliferation effects on human chondrosarcoma cells [26]. Treatment of TGFβ-1 (3–30 ng/ml) only slightly increased the cell proliferation by using MTT assay (data not shown). To examine the migration activity by TGF-β1, the number of migrating cells in each experiment was adjusted by the cell viability assay to

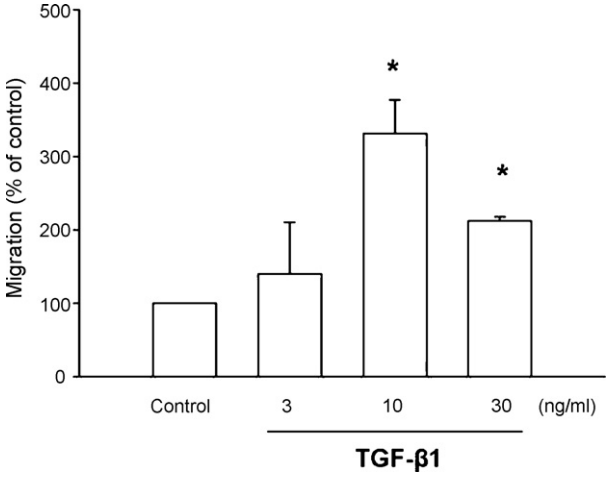

Fig. 1 – TGF-β1-induced the migration activity of human chondrosarcoma cells JJ012 cells were incubated with various concentrations of TGF-β1, and *in vitro* migration activities measured with the Transwell after 24 h showed all supported the JJ012 cell migrations in a dose-dependent way. Results are expressed as the mean ± S.E. * $p < 0.05$ compared with control.

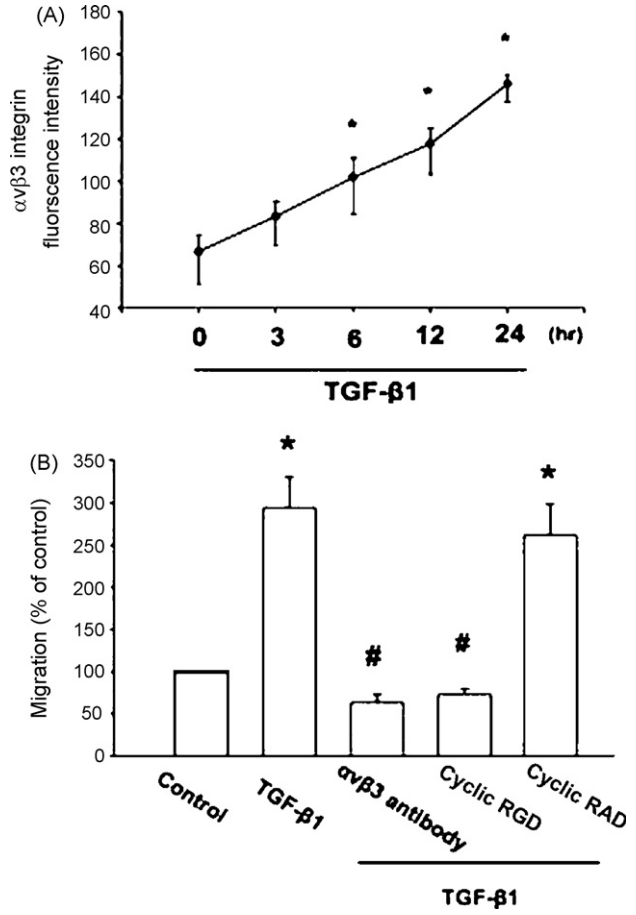

Fig. 2 – TGF-β1-directed migration activity of human chondrosarcoma cells involves upregulation of αvβ3 integrins. Cells were incubated with TGF-β1 (10 ng/ml) for various time intervals, and the cell surface expression of αvβ3 integrin was determined using flow cytometer (A). JJ012 cells were pretreated with αvβ3 monoclonal antibody (10 μg/ml), cyclic RGD (100 nM), cyclic RAD (100 nM) for 30 min followed by stimulation with TGF-β1 (10 ng/ml). The *in vitro* migration activity measured after 24 h showed that αvβ3 monoclonal antibody and cyclic RGD but not cyclic RAD could inhibit the activities (B). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with TGF-β1-treated group.

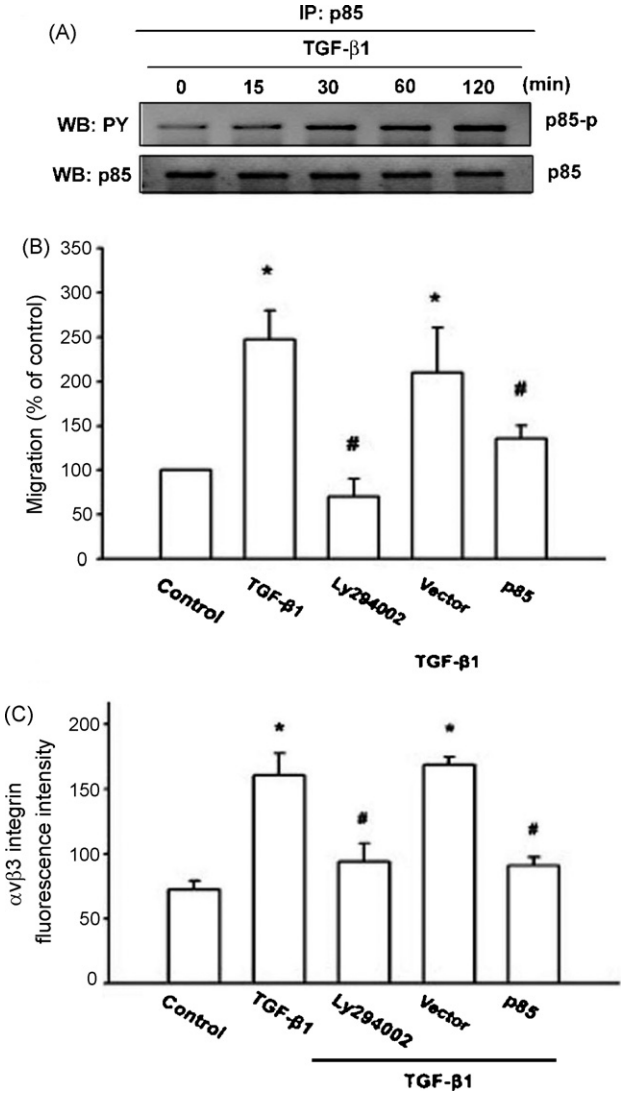

Fig. 3 – PI3K is involved in TGF-β1-mediated migration and integrin upregulation in human chondrosarcoma cells. (A) Cells were incubated with TGF-β1 (10 ng/ml) for indicated time intervals, and cell lysates were immunoprecipitated (IP) with an antibody specific for p85. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted (WB) with anti-phosphotyrosine (PY). (B) Cell were pretreated for 30 min with Ly204002 (10 μM) or transfected with dominant negative (DN) mutant of p85 for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C) Cell were pretreated for 30 min with Ly294002 (10 μM) or transfected with DN-mutant of p85 for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and the cell surface αvβ3 integrin was measured by using flow cytometry. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with TGF-β1-treated group.

correct for proliferation effects of the TGF-β1 treatment (corrected migrating cell number = counted migrating cell number/percentage of viable cells). TGFβ-1 directed human chondrosarcoma cells (JJ012 cell) migration (Fig. 1). Previous studies have shown significant expression of integrin in human cancer cells [28,29]. We therefore, hypothesized that integrins may be involved in TGFβ-1-directed chondrosarcoma cells migration. Flow cytometry analysis showed that TGFβ-1-induced the cell surface expression of αvβ3 integrin time-dependently (Fig. 2A). Pretreatment of cells for 30 min with anti-αvβ3 monoclonal antibody (mAb) (10 μg/ml) markedly inhibited the TGFβ-1-induced cancer migration (Fig. 2B). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind αvβ3 at high affinity and block its function effectively at low concentrations [30]. Pretreatment of cells for 30 min with cyclic RGD but not cyclic RAD inhibited the TGFβ-1-induced cell migration (Fig. 2B). These data suggest that TGFβ-1-induced cancer migration may occur via activation of αvβ3 integrin receptor.

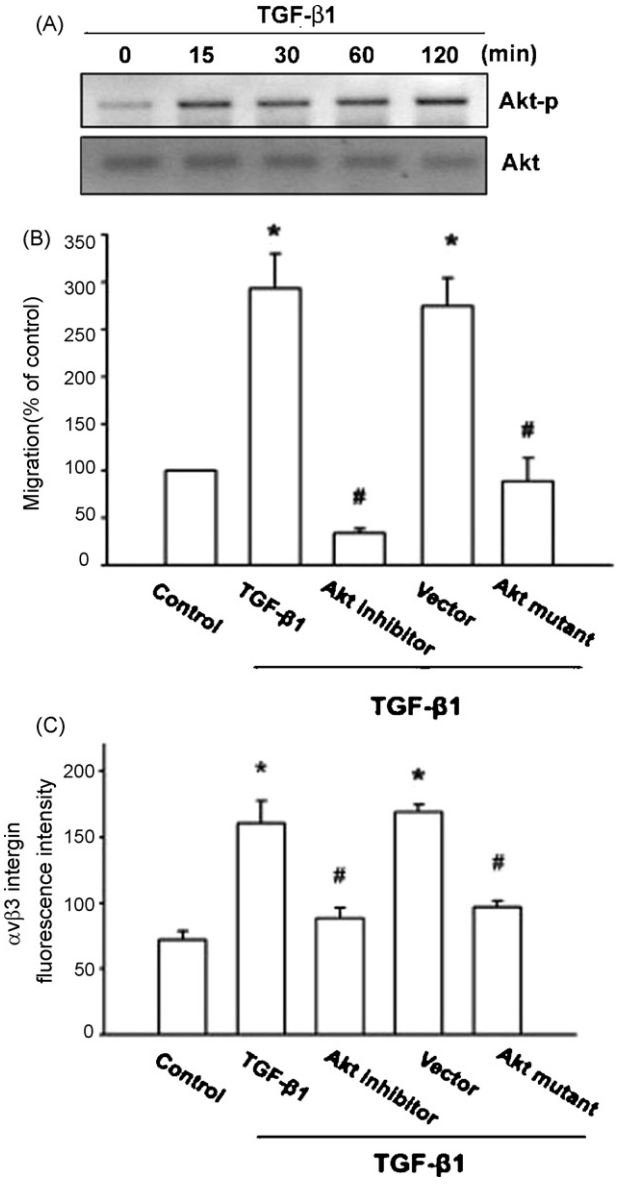

Fig. 4 – Akt is involved in TGF-β1-mediated migration and integrin upregulation in human chondrosarcoma cells. (A) Cells were incubated with TGF-β1 (10 ng/ml) for indicated time intervals, and p-Akt expression was determined by Western blot analysis. TGF-β1 activated the Akt pathway in JJ012 cells. (B) Cell were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with dominant negative (DN) mutant of Akt for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C) Cell were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with DN-mutant of Akt for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and the cell surface αvβ3 integrin was measured by using flow cytometry. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with TGF-β1-treated group.

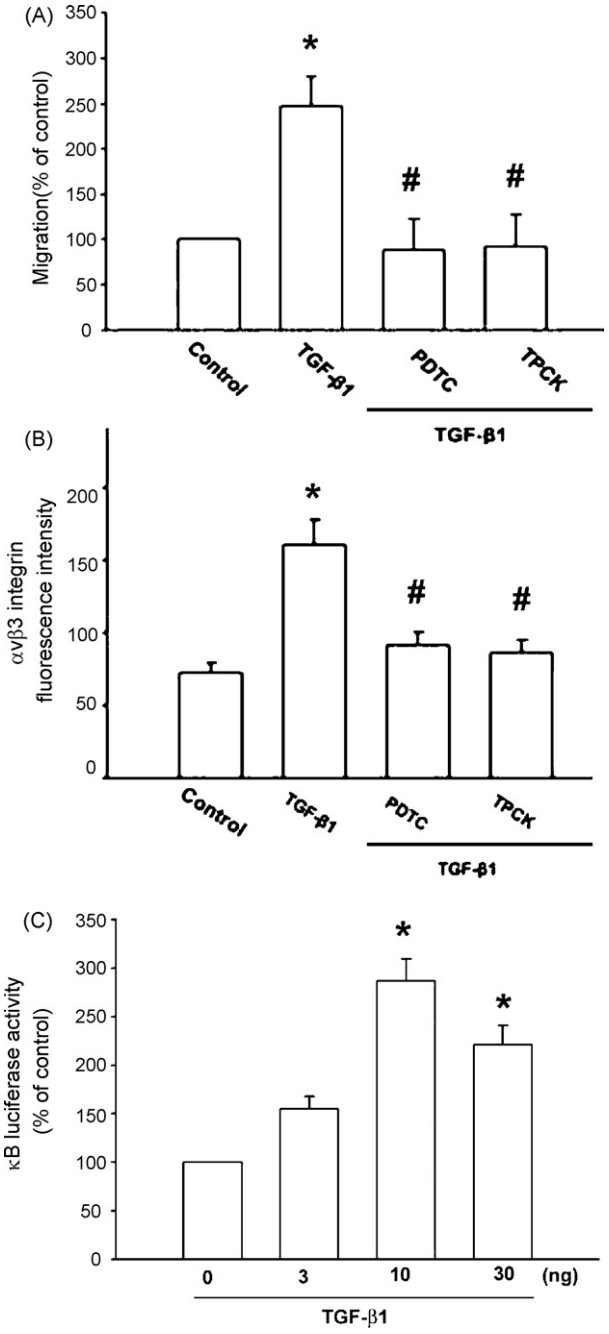

Fig. 5 – TGF-β1 induces cells migration and integrin upregulation through NF-κB. (A) Cell were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with TGF-β1 (10 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (B) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with TGF-β1 (10 ng/ml) for 24 h, and the cell surface αvβ3 integrin was measured by flow cytometry. (C) Cells were transfected with κB promoter plasmid for 24 h, and were then incubated with TGF-β1 for 24 h. 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 TGF-β1-treated group.

3.2. PI3K and Akt signaling pathways are involved in the TGFβ-1-mediated integrin upregulation and migration of chondrosarcoma cells

PI3K/Akt can be activated by a variety of growth factors, such as insulin, nerve growth factors, and TGF-β1 [31,32]. We then examined whether TGF-β1 stimulation also enhances the activation PI3K. Stimulation of JJ012 cells led to a significant increase of phosphorylation of p85 subunit of PI3K, as assessed by the measurement of phosphotyrosine from immunoprecipitated lysates using p85 (Fig. 3A). TGF-β1-induced the migration and αvβ3 integrin expression of JJ012 cells were greatly reduced by treatment with Ly294002 (10 μM), a specific PI3K inhibitor (Fig. 3B and C). In addition, transfection of cells with p85 mutant also inhibited TGF-β1-induced the migration and integrin up-regulation of chondrosarcoma cells (Fig. 3B and C). Ser^{473} residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation [33]. To examine the crucial role of PI3K/Akt in cancer migration and integrin up-regulation, we determined Akt Ser^{473} phosphorylation in response to TGF-β1. As shown in Fig. 4A, treatment of JJ012 cells with TGF-β1 resulted in time-dependent phosphorylation of Akt Ser^{473}. Pretreatment of cells with Akt inhibitor (10 μM) or transfection with Akt mutant antagonized the TGF-β1-induced the migration and αvβ3 integrin expression of JJ012 cells (Fig. 4B and C).

3.3. NF-κB signaling pathways is involved in the TGF-β1-mediated integrin upregulation and migration activity

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [34]. To examine whether NF-κB activation is involved in TGFβ-1-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 5A shows that JJ012 cells pretreated with PDTC (10 μM) and inhibited TGFβ-1-induced chondrosarcoma cell migration. Furthermore, JJ012 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced TGFβ-1-induced cancer cell migration (Fig. 5A). Treatment of cells with anti-αvβ3 mAb, cyclo-RGDfV, Ly294002, Akt inhibitor, PDTC or TPCK did not affect the cell viability by using MTT assay (data not shown). In addition, treatment of cells with PDTC or TPCK also antagonized TGFβ-1-induced the expression of αvβ3 integrins (Fig. 5B). To directly determine NF-κB activation after TGFβ-1 treatment, JJ012 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 5C, TGFβ-1 (3–30 ng/ml) treatment of JJ012 cells for 24 h caused a concentration-dependent increase in κB-luciferase activity. These results indicated that NF-κB activation is important for TGFβ-1-induced cancer cell migration and the expression of αvβ3 integrins. We further examined the upstream molecules involved in TGFβ-1-induced NF-κB activation. Stimulation of cells with TGFβ-1-induced IKKα/β

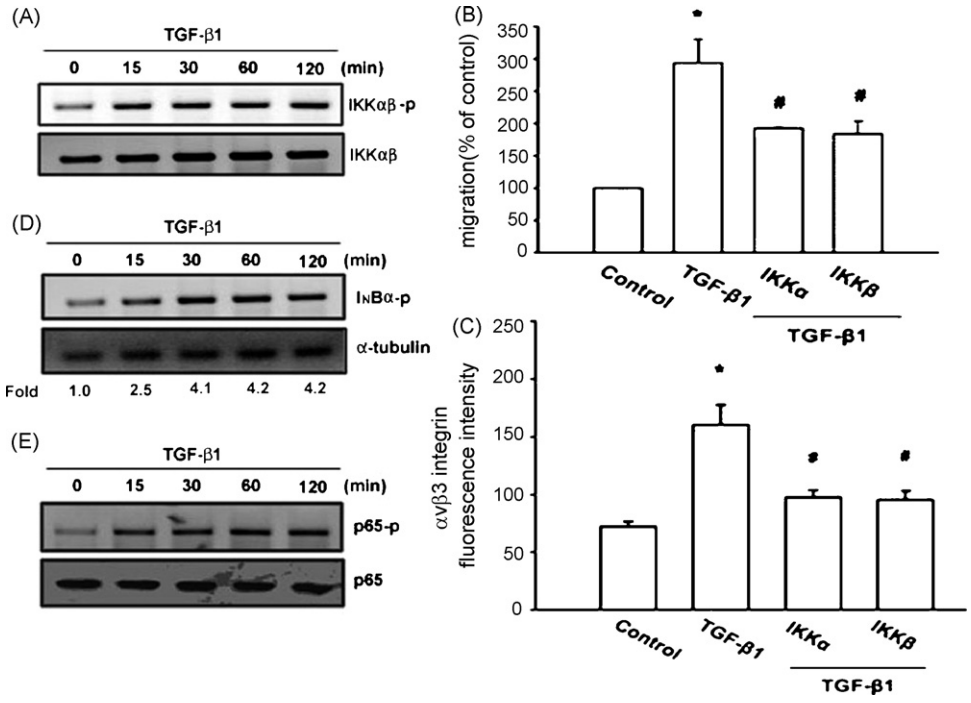

Fig. 6 – TGF-β1 induces IKKα/β phosphorylation, IκBα phosphorylation and p65 Ser^{536} phosphorylation in chondrosarcoma cells. (A) Cells were incubated with TGF-β1 (10 ng/ml) for indicated time intervals, and p-IKKα/β expression was determined by Western blot analysis. (B) Cell were transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C) Cell were transfected with DN mutant of IKKα or IKKβ for 24 h followed by stimulation with TGF-β1 (10 ng/ml), and the cell surface αvβ3 integrin was measured by flow cytometry. (D and E) Cells were incubated with TGF-β1 (10 ng/ml) for indicated time intervals, and p-IκBα and p65 Ser^{536} expression was determined by Western blot analysis. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with TGF-β1-treated group.

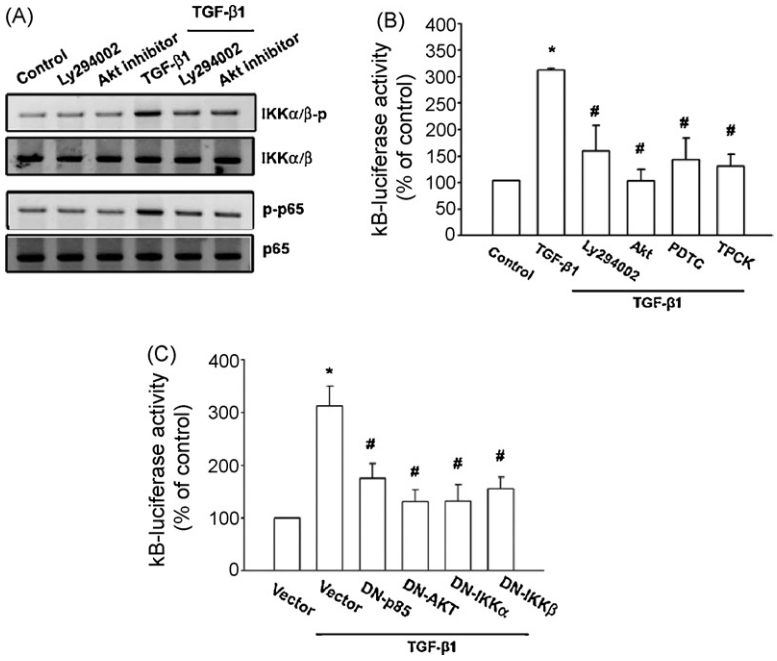

Fig. 7 – PI3K/Akt involved the TGF-β1-induced IKKα/β phosphorylation, p65 Ser^{536} phosphorylation and κB-luciferase activity in chondrosarcoma cells. (A) JJ012 cells were pretreated with Ly294002 (10 μM), Akt inhibitor (10 μM) for 30 min before treatment with TGF-β1 (10 ng/ml) for another 120 min, after which IKKα/β phosphorylation, p65 Ser^{536} phosphorylation were determined by immunoblotting with antibodies specific for phospho-IKKα/β and phospho-p65, respectively. Equal loading in each lane is shown by the similar intensities of IKKα/β and p65, respectively. (B and C) JJ012 cells transiently transfected with κB-luciferase plasmid for 24 h were either cotransfected with p85, Akt, IKKα and IKKβ mutants or pretreated with Ly294002 (10 μM), Akt inhibitor (10 μM), PDTC (10 μM) or TPCK (3 μM) for 30 min, before incubation with TGF-β1 (10 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 ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with TGF-β1-treated group.

phosphorylation in a time-dependent manner (Fig. 6A). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the TGFβ-1-induced cancer cells migration and integrin up-regulation (Fig. 6B and C). These data suggest that IKKα/β activation is involved in TGFβ-1-induced the migration activity of human chondrosarcoma cells. Treatment with chondrosarcoma cells with TGFβ-1 also caused IκBα phosphorylation in a time-dependent manner (Fig. 6D). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [35,36], and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of JJ012 cells with TGFβ-1 for various time intervals resulted in p65 Ser^{536} phosphorylation which began at 15 min and was sustained to 120 min (Fig. 6E, upper panel). The protein levels of p65 were not affected by TGFβ-1 treatment (Fig. 6E, bottom panel).

3.4. *PI3K/Akt signal transduction-mediated TGFβ-1-induced IKKα/β phosphorylation, p65 phosphorylation and κB-luciferase activity*

To further investigate whether TGFβ-1-induced IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, and NF-κB activation occur through the PI3K/Akt pathway, JJ012 cells were pretreated for 30 min with Ly294002 (10 μM) and Akt inhibitor (10 μM), which inhibited the TGFβ-1-induced increase in IKKα/β phosphorylation as shown in Fig. 7A. Moreover, the TGFβ-1-induced increase in p65 Ser^{536} phosphorylation was also attenuated by Ly294002 and Akt inhibitor (Fig. 7A). In addition, the TGFβ-1-induced increase in κB-luciferase activity was also inhibited by treatment with Ly294002 and Akt inhibitor (Fig. 7B). Co-transfection with p85 and Akt mutants also reduced the TGFβ-1-induced κB-luciferase activity (Fig. 7C). Taken together, these data suggest that activation of PI3K/Akt is required for TGFβ-1-induced IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, and NF-κB activation in chondrosarcoma cells.

4. Discussion

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 [37]. Integrins play particularly pivotal roles in cell migration and adhesion. Integrins consist of two transmembrane subunits; an α subunit and a β subunit. In mammals, 18α and 8β subunits associate in various combinations to form 24 integrins that can bind to various

ECM ligands [38]. Previous studies have shown that TGF-β1 modulate cell migration and invasion in several cancer cells [19,27]. However, the expression of integrins by TGF-β1 in human chondrosarcoma cells is mostly unknown. Here we found that TGF-β1 increased αvβ3 integrins expression by using flow cytometry analysis, which is play an important role during tumor migration. In the present study, we used αvβ3 integrin antibody to determine the role of αvβ3 integrin and found that it inhibited TGF-β1-induced cell migration, indicating the possible involvement of αvβ3 integrin in TGF-β1-induced migration in chondrosarcoma cells. This was further confirmed by the result that the cyclic RGD but not cyclic RAD inhibited the enhancement of migration activity by TGF-β1, indicating the involvement of αvβ3 integrin in TGF-β1-mediated induction of chemomigration. On the other hand, the highest dose (TGF-β1; 30 ng/ml) does not appear to be significantly greater than the lowest dose (3 ng/ml) in migration activity and κB-luciferase activity (Figs. 1 and 5C). Therefore, the highest dose TGB-β1 may down-regulation some signaling molecules or reducing cell viability 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. The PI3K/Akt pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [39,40]. Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K. We found TGF-β1-enhanced p85 subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 antagonized the increase of migration and integrin expression by TGF-β1 stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of migration and integrin expression by TGF-β1. Moreover, we also found that TGF-β1 activated Akt Ser^{473} phosphorylation, while Akt inhibitor and Akt mutant inhibited TGF-β1-mediated chemomigration. Our data indicates that PI3K/Akt might play an important role in the expression of integrin and migration of human chondrosarcoma cells.

Many NF-κB activation pathways have been reported, and all of them rely on sequentially activated kinase cascades [36]. The classical pathway is triggered by various proinflammatory cytokines such as IL-1β and TNF-α [36]. 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 [36]. 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 [36]. p65 Ser^{276} is phosphorylated by the protein kinase A catalytic subunit and mitogen- and stress-activated protein kinase-1, and this phosphorylation increases p65 transcriptional activity [41,42]. In addition, 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 [43]. The results of this study showed that the PI3K/Akt pathway contributed to TGF-β1-induced p65 Ser^{536} phosphorylation in JJ012 cells. TGF-β1-induced IKKα/β phosphorylation as well as an increase in p65 phosphorylation at Ser^{536} which began at 15 and 120 min, respectively, and Ly294002 and Akt inhibitor inhibited TGF-β1-induced p65 phosphorylation at Ser^{536}. These results indicate that PI3K/Akg may act through IKKα/β to increase p65 phosphorylation at Ser^{536} and enhance NF-κB transactivation. Often, NF-κB signaling is rapid and transient. In present study, we found TGF-β1-induced IκBα and p65 phosphorylation which began at 15 min and sustained to 120 min (Fig. 6). These levels are back to baseline by 4 h after TGF-β1 stimulation (data not shown). The similar data have been reported in SDF-1 and leptin-induced IκBα and p65 phosphorylation [45,47]. In addition, we also found that TGF-β1 (10 ng/ml) increased the migration of the other chondrosarcoma cells (SW 1353). Treatment of the cells (SW 1353) with cyclic RGD peptide, Ly294002, Akt inhibitor, PDTC and TPCK all antagonized the TGF-β1-increaed the migration activity (data not shown). Therefore, the same signaling pathway was required for TGF-β1-induced cell migration in human chondrosarcoma cells.

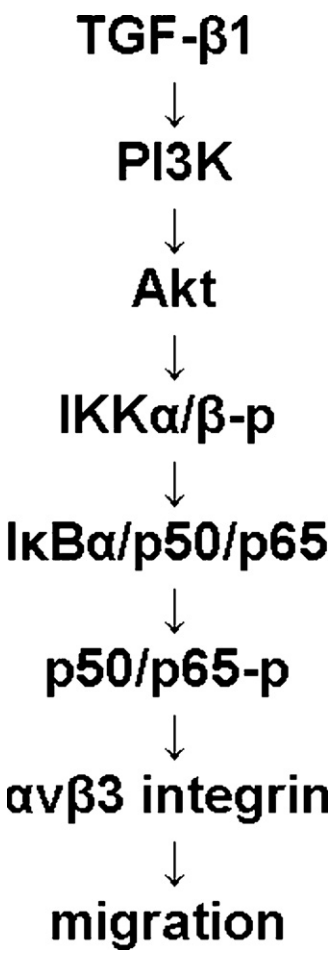

Fig. 8 – Schematic presentation of the signaling pathways involved in TGF-β1-induced migration and integrins expression of chondrosarcoma cells. TGF-β1 activates PI3K and Akt pathway, which in turn induces IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, and NF-κB activation, which leads to αvβ3 integrins expression and increases the migration of human chondrosarcoma cells.

In conclusion, we present here a novel mechanism of TGF-β1-directed migration of chondrosarcoma cells by upregulation of αvβ3 integrins. TGF-β1 increases cells migration and integrins expression by activation of PI3K, Akt, IKKα/β, and NF-κB-dependent pathway (Fig. 8).

Acknowledgments

This work was supported by grants from the National Science Council of Taiwan (96-2815-C-039-034-B; 96-2320-B-039-028-MY3) and China Medical University (CMU96-097). We thank Dr. R.H. Chen for providing p85 and Akt mutants; Dr. H. Hakano for providing IKKα and IKKβ mutants.

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