Integrin-β1 regulates chondrocyte proliferation and apoptosis through the upregulation of GIT1 expression

LONG-QIANG ZHANG1,2*, GUANG-ZONG ZHAO3*, XIAO-YAN XU3, JUN FANG2, JING-MING CHEN2, JI-WEN LI2, XUE-JIAN GAO4, LI-JUAN HAO5 and YUN-ZHEN CHEN1

1Department of Orthopedics, Qilu Hospital of Shandong University, Jinan, Shandong 250012; 2Department of Orthopedics, Yidu Central Hospital of Weifang, Weifang, Shandong 262500; 3Department of Oncology, Qingzhou Hospital of Traditional Chinese Medicine, Qingzhou, Shandong 262500; 4Department of Orthopedics, The 89th Hospital of PLA, Weifang, Shandong 261021; 5Department of Urologic Surgery, Yidu Central Hospital of Weifang, Weifang, Shandong 262500, P.R. China

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Abstract. Chondrocytes play a critical role in the repair process of osteoarthritis, which is also known as degenerative arthritis. Integrins, as the key family of cell surface receptors, are responsible for the regulation of chondrocyte proliferation, differentiation, survival and apoptosis through the recruitment and activation of downstream adaptor proteins. Moreover, G-protein-coupled receptor kinase interacting protein-1 (GIT1) exerts its effects on cell proliferation and migration through interaction with various cytokines. It has been previously suggested that GIT1 acts as a vital protein downstream of the integrin-mediated pathway. In the present study, we investigated the effects of integrin-β1 on cell proliferation and apoptosis, as well as the underlying mechanisms in chondrocytes in vitro. Following transfection with a vector expressing integrin-β1, our results revealed that the overexpression of integrin-β1 enhanced GIT1 expression, whereas the knockdown of integrin-β1 by siRNA suppressed GIT1 expression. However, no significant effect was observed on integrin-β1 expression following the enforced overexpression of GIT1, which suggests that GIT1 is localized downstream of integrin-β1. In other words, integrin-β1 regulates the expression of GIT1. Furthermore, this study demonstrated that integrin-β1 and GIT1 increased the expression levels of aggrecan and type II collagen, thus promoting chondrocyte proliferation; however, they inhibited chondrocyte apoptosis. Taken together, our data demonstrate that integrin-β1 plays a vital role in chondrocyte proliferation, differentiation and apoptosis. GIT1 exerts effects similar to those of integrin-β1 and is a downstream target of integrin-β1.

Introduction

Poorly treated or untreated articular cartilage defects easily give rise to osteoarthritis (OA), which is a progressive degradation of articular cartilage, resulting in the loss of joint function (1,2). Cartilage cells play a crucial role in the repair process of OA. The investigation of the mechanisms responsible for the stimulation of chondrocyte proliferation and the suppression of chondrocyte apoptosis in vitro is of clinical significance. It has been previously demonstrated that a variety of cytokines, hormones, modifiers and drugs affect the biosynthesis of chondrocytes in vitro experiments (3).

The interaction between chondrocytes and their surrounding extracellular matrix (ECM) has significant effects on the metabolic homeostasis of the articular cartilage (integrin-β1) (4,5). The ECM molecules, such as collagen, hyaluronan and aggrecan, are the most abundant macromolecules found in cartilage (3-5). Additionally, the overexpression of collagen and aggrecan has been found in the early stages of OA (1,2). Integrins, cell surface adhesive proteins, are expressed in the majority of cells of multicellular animals, such as human chondrocytes (4,5). Integrins are characterized by participating in cell-cell and cell-matrix communications. As transmembrane glycoprotein receptors, integrins also contribute to the cell attachment and cell signaling pathways. For instance, these adhesion proteins may affect bi-directional signaling in cells, affecting processes, such as cytoskeletal arrangement, growth factor signaling and gene transcription. Moreover, integrins mediate cell development, the immune response and diseases, such as cancer and autoimmune disorders. Integrins, as heterodimeric transmembrane glycoproteins, are made up of α and β chains, being non-covalently bonded protein complexes (4,5). The integrin-β1 subfamily includes a variety of receptors, such as fibronectin, collagens and laminin for ECM proteins. The expression of target genes and the integrin expression and affinity may be altered by integrin binding due
to cell signaling pathway stimulation (5,6). Therefore, although the role of integrins in chondrocytes is not yet completely understood, it is believed that integrins exert significant effects on chondrocytes.

G-protein-coupled receptor kinase interacting protein-1 (GIT1) was originally identified by a yeast two-hybrid screen and is widely present in mammals and birds (7,8). It is a scaffold protein, acting as a GTPase-activating protein for the ADP-ribosylation factor family of proteins (7-10). GIT1 is mainly located in cell adhesion and cytoplasmic complex structures and stimulates the activation of and the membrane recruitment of G-protein-coupled receptor kinase 2 (GRK2) (7). Initially, the identified function of GIT1 was to regulate cell surface G-protein-coupled receptors in cell transduction pathways (8). Later findings demonstrated that GIT1 plays a vital role in cell proliferation and migration through interaction with a variety of cytokines, including ARF, Rac1, Cdc42, GTPases, PAK, PIX, MEK1, PLC and paxillin (9,10).

Thus far, it has been suggested that GIT1 exerts significant effects on chondrocytes. In the study by Menon et al (11), for example, GIT1 underwent Src-mediated tyrosine phosphorylation and was found to be a key component in phospholipase Cγ2 (PLCγ2) phosphorylation, suggesting that it was a regulator of the receptor activator of NF-κB (RANK) signaling. Their results also demonstrated that GIT1 has great potential for use in the treatment of osteoporosis, due to its ability to regulate osteoclast function and bone mass (11). However, current studies on GIT1 in bone cells focus on the migration of osteoblasts. In the study by Ren et al (12), it was demonstrated that the phosphorylation of GIT1 tyrosine 321 is necessary for the association with focal adhesion kinase (FAK) and for the migration of osteoblasts induced by platelet-derived growth factor (PDGF). The results from the study by Liu et al (13) demonstrated that GIT1 and integrin αββ8, in association with Pak-interacting exchange factor-β (PIX), had an effect on the regulation of vascular stability, cerebral angiogenesis and endothelial cell proliferation in the developing embryo. Furthermore, the data reported in the study by Sato et al (14) revealed that Src kinases transfer Rac-GEF/PIX to the vicinity of the integrin through the tyrosine phosphorylation of GIT1, resulting in the regulation of the cytoskeletal reorganization downstream of α(IIb)β3. As cell surface adhesion receptors, integrins are able to respond to a variety of stimuli from the extracellular environment in an attempt to regulate cell activity and function (6). Integrins, including αβ1, α3β1, α10β1 and αβ5, are mainly expressed in the articular cartilage in healthy adults (15,16). In addition, the study by Ren et al (17) provided evidence that periodic mechanical stress induces the activation of the integrin-β1-Src-PLCγ1/Rac-mediated extracellular signal-regulated kinase 1/2 (ERK1/2) mitogenic signaling pathway, enhancing chondrocyte proliferation and matrix synthesis. Taken together, these data suggest that there is a close association between GIT1 and integrins in bone cells. Therefore, the aim of this study was to examine the role of GIT1 and integrins (integrin-β1 in particular) in chondrocytes.

Materials and methods

Cell culture. The dissection of 1-week-old Sprague-Dawley (SD) neonatal rats (Institute of Laboratory Animal Sciences, CAMS&PUMC, Beijing, China) was performed in order to obtain limb joints under sterile conditions. Approximately 1 mm² sections of cartilage were cut from the transparent central portion of the articular cartilage surface. The tissue sections were digested by 0.25% trypsin (Gibco, Beijing, China) at 37°C for 30 min, and then centrifugation was performed at 1,400 rpm for 5 min to collect the pellets. Following digestion of the pellets by 0.2% type II collagenase (Sigma, Beijing, China) at 37°C for 4 h, the solution was filtered using a 200-mesh filter and centrifuged at 1,400 rpm for 5 min. Furthermore, Dulbecco’s modified Eagle’s medium (DMEM)-F12 with 10% fetal bovine serum (FBS) (both from Gibco), penicillin (100 U/ml) and streptomycin (50 U/ml) were used to maintain the cells at 37°C in a humidified incubator with 5% CO₂ atmosphere. An inverted phase contrast microscope (CKX41-A32PH; Olympus, Shanghai, China) was used to observe the morphology of the purified cells after repeated isolation and culture. Subsequently, the cells were passed by 0.25% trypsin due to growing fully in the dish. The study protocol conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1985) and was approved by the Animal Care and Use Committee, Qilu Hospital of Shandong University, Jinan, China.

Vector construction and transfection with siRNA. RNA from chondrocytes was extracted using TRIzol reagent (Invitrogen, Shanghai, China). Following reverse transcription, polymerase chain reaction (PCR) was used to amplify the integrin-β1 and GIT1 coding regions. They were then cloned into the pcDNA3.1 vector (Promega, Beijing, China) through the KpnI and EcoRI (Takara, Dalian, China) restriction enzymes. The gene amplification primers are listed in Table I. Subsequently, 1×10⁶ cells/ml cells were seeded in 6-well plates and incubated for 24 h. Plasmid and siRNA transfection was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions, when the cell confluence reached approximately 70%. Integrin-β1 siRNA (target sequence 5′-AAA AGT CTT GGA ACA GAT CTG-3′) and negative control (NC) siRNA (HP GenomeWide siRNA; Qiagen, Germany) were prepared and transfected at 100 nM for 48 h as previously described (18). Subsequently, the transfected cells were analyzed by quantitative PCR and western blot analysis.

Quantitative fluorescence PCR. The cells were collected, and total RNA was extracted using TRIzol reagent. Reverse transcription was performed with 1 μg of RNA following quantification. Quantitative PCR was conducted using SYBR-Green (Toyobo, Osaka, Japan) with 100 ng of cDNA in a 20 µl system. The primer sequences are listed in Table I. The reaction conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. All reactions were run in triplicate, and quantitative analysis was performed by comparing the 2ΔΔCt values.

Western blot analysis. Total cellular protein was extracted from the cells using lysis buffer. The supernatant containing proteins was obtained after shaking at 4°C for 20 min and centrifugation at 14,000 rpm for 4°C for 10 min. The Bradford protein assay (Pierce, Rockford, IL, USA) was used to determine the protein concentration. An equal amount of proteins
was loaded on 8% glycine gels [sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE)]. Following electrophoresis, the separate proteins were transferred onto nitrocellulose membranes (Pierce). Subsequently, 5% non-fat milk (Pierce) in TBST buffer was used to block the membranes for 1 h. Subsequently, the membranes were incubated with GIT1 (#9699; Cell Signaling Technology), aggrecan (sc-25674; Santa Cruz Biotechnology, Santa Cruz, CA, USA), type II collagen (sc-28887; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (clone 4G10; Sigma) primary antibodies at 1:1,000 dilution overnight at 4°C. The membranes were washed with TBST 3× and incubated with 0.2% Triton X-100 for 5 min. Following incubation with 0.2% Triton X-100, the membranes were fixed with 4% formaldehyde at 4°C for 25 min. The cells were incubated with 0.2% Triton X-100 for 5 min. Following incubation with 100 µl equilibration buffer at room temperature for 10 min, the cells were mixed with 50 µl TUNEL reaction mixture containing nucleotide mixture and terminal deoxynucleotidyl transferase (TdT) for 60 min at 37°C. Subsequently, 2X saline sodium citrate (SSC) was used to wash the cells for incubation at 1 h 37°C. TBST was used for washing the membranes every 10 min, for a total of 30 min. Protein bands were detected by ECL (Pierce). The light-emitting film was scanned using a gel imaging system (UVP Co., Upland, CA, USA) of western blot analysis and gray values of the band were measured using Gel-Pro Analyzer software (Media Cybernetics, Inc., Silver Spring, MD, USA).

**BrdU cell proliferation assay.** BrdU assay was used to investigate the role of integrin-β1 and GIT1 in cell proliferation. Briefly, the cultured cells were seeded in 6-well plates and incubated for 24 h, and then plasmid and siRNA transfection was carried out using Lipofectamine 2000. A BrdU colorimetric immunoassay kit (Cell Proliferation ELISA; Roche Diagnostics GmbH, Mannheim, Germany) was used for the quantification of cell proliferation according to the protocol provided by the manufacturer. Cell proliferation was expressed as the mean percentage of the control values (set at 100%).

**TUNEL-DAPI co-staining assay.** TUNEL-DAPI co-staining assay with the In Situ Cell Death Detection kit (Roche Diagnostics GmbH) was used for measuring cell apoptosis. Briefly, after being washed with phosphate-buffered saline (PBS), the cultured cells were fixed with 4% formaldehyde at 4°C for 25 min. The cells were incubated with 0.2% Triton X-100 for 5 min. Following incubation with 100 µl equilibration buffer at room temperature for 10 min, the cells were mixed with 50 µl TUNEL reaction mixture containing nucleotide mixture and terminal deoxynucleotidyl transferase (TdT) for 60 min at 37°C. Subsequently, 2X saline sodium citrate (SSC) was used to wash the cells for incubation at 1 h 37°C. TBST was used for washing the membranes every 10 min, for a total of 30 min. Protein bands were detected by ECL (Pierce). The light-emitting film was scanned using a gel imaging system (UVP Co., Upland, CA, USA) of western blot analysis and gray values of the band were measured using Gel-Pro Analyzer software (Media Cybernetics, Inc., Silver Spring, MD, USA).

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**Statistical analysis.** The experiments were carried out at least in triplicate, and the results are expressed as the means ± SD. The SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Differences between 2 groups were analyzed using a two-tailed Student’s t-test and those between 3 or more groups by one-way ANOVA multiple comparisons. Values of P<0.05 or P<0.01 were considered to indicate statistically significant differences.

**Results**

**Integrin-β1 increases the expression of GIT1.** In the present study, siRNA silencing techniques, quantitative PCR and western blot analysis were used to analyze the role of integrin-β1, GIT1 and their crosstalk in cartilage cells. The cells were seeded in 6-well plates and incubated for 24 h, and then siRNA transfection was carried out using Lipofectamine 2000. Subsequently, quantitative PCR and western blot analysis were carried out to detect the mRNA and protein expression levels. Following transfection, the overexpression of integrin-β1 was found to promote GIT1 expression, whereas the silencing of integrin-β1 by siRNA inhibited the expression of GIT1 (Fig. 1). However, GIT1 overexpression
had no significant effect on the expression of integrin-β1 (Fig. 1). These results suggest that integrin-β1 regulates the expression of GIT1 and is located upstream of GIT1.

Figure 1. The expression of G-protein-coupled receptor kinase interacting protein-1 (GIT1) is promoted by integrin-β1. The cells were seeded in 6-well plates and incubated for 24 h, and then integrin-β1 siRNA transfection was carried out using Lipofectamine 2000. Following siRNA transfection, the expression of integrin-β1 and GIT1 was measured by (A) quantitative fluorescence PCR and (B and C) western blot analysis. The experiments were carried out at least in triplicate, and the results are expressed as the means ± SD, *P<0.05 vs. the control.

**Integrin-β1 and GIT1 promote chondrocyte proliferation.** The effects of integrin-β1 and GIT1 on chondrocyte proliferation were also investigated. Briefly, the cells were seeded into 6-well plates and incubated for 24 h, and then integrin-β1-pcDNA3.1, GIT1-pcDNA3.1 and integrin-β1 siRNA transfection was carried out using Lipofectamine 2000. BrdU assay was used to assess the proliferation of cartilage cells. The results revealed that the overexpression of integrin-β1 and GIT1 enhanced chondrocyte proliferation, although the effects of integrin-β1 overexpression on chondrocyte proliferation were more prominent (Fig. 2). In other words, integrin-β1 plays an essential role in the regulation of chondrocyte proliferation. When integrin-β1 expression was depleted using siRNA, chondrocyte proliferation was significantly inhibited. These results suggest that integrin-β1 plays a role in chondrocyte proliferation.

**Integrin-β1 and GIT1 affect the synthesis of ECM in chondrocytes.** It is well known that aggrecan and type II collagen are abundant in cartilage ECM (3-5); thus, the synthesis of the ECM can be determined using them as references. As a result, the activity and function of tissue engineering are reflected by chondrocyte proliferation and the gene expression levels of type II collagen and aggrecan. Western blot analysis was used to investigate the protein expression levels. The expression levels of aggrecan and type II collagen were significantly enhanced by the overexpression of integrin-β1 and GIT1 (Fig. 3). However, the effects of integrin-β1 on the stimulation of the expression of aggrecan and type II collagen were much more prominent than...
Those of GIT1 (Fig. 3). On the other hand, the expression levels of aggrecan and type II collagen were significantly reduced when integrin-β1 expression was depleted using specific siRNA (Fig. 3). Therefore, these results demonstrate that integrin-β1 regulates aggrecan and type II collagen expression.

Integrin-β1 inhibits chondrocyte apoptosis. Programmed cell death (PCD), known as apoptosis, is a crucial process in multicellular organisms. Chondrocyte apoptosis plays a vital role in bone remodeling. In the present study, in order to further examine the role of integrin-β1 in cartilage cells, TUNEL-DAPI assay was used to measure DNA fragmentation resulting from apoptotic signaling cascades. For the purpose of understanding the role of integrin-β1 in chondrocyte apoptosis, TUNEL assay was used to identify the presence of nicks in the DNA. DNA fragmentation, which is associated with apoptotic events, was observed in the chondrocytes (Fig. 4). The results revealed that the occurrence of apoptotic cell death was significantly suppressed by the overexpression of integrin-β1 (Fig. 4). Moreover, following the silencing of integrin-β1 using specific siRNA, there were no significant effects observed on cell apoptosis (Fig. 4). These results suggest that integrin-β1 is required for the survival of chondrocytes.

Discussion

Integrins are a key family of cell surface adhesion receptors regarded as heterodimeric transmembrane glycoproteins (19). It has been demonstrated that integrins are widely expressed in cells. There are several integrins located on the cell surface of the majority the cells, playing a critical role in a variety of metabolic activities (19,20). In fact, integrins without any kinase activity regulate the recruitment and activation of the downstream adapter protein of integrin signaling, resulting in intracellular signal transduction (18,19).

Integrins function as a bridge connecting chondrocytes and ECM, regulating the interaction between the two, thus affecting the differentiation, proliferation, growth and apoptosis of cartilage cells and ECM structure (3-5). It is known that integrins, including integrins α1β1, α2β1, α3β1, α5β1, α6β1, α10β1, αVβ3 and αVβ5 in cartilage cells play a role in mechanical signal transduction (of which integrins α1β1, α3β1, α5β1 play the most prominent role) and that integrin α10β1 is a main molecule for stabilizing the chondrocyte phenotype (18). Integrin-β1 is responsible for cartilage tissue repair and remodeling by binding and assembling important structural ECM proteins, such as collagen. For instance, integrins β1l and α2βl have been found to mediate chondrocyte adhesion to type II collagen and laminin (3-5,18). However, integrins α3β1 and α5β1 mediate the adhesion of cartilage cells to type II collagen and fibronectin (19). In addition, Schagemann et al (20) found that biological materials can promote chondrocyte CD44 and integrin-β1 expression, being conducive to cartilage repair and reconstruction in the investigation of tissue engineered cartilage.

In the present study, we demonstrated that the upregulation of integrin-β1 promoted chondrocyte proliferation and reduced apoptosis. Moreover, in the study by Wang et al (21), it was suggested that integrins play an essential role in the regulation of chondrocyte proliferation and differentiation, and that...
RhoA is characterized as the downstream target of integrin molecules. Fibroblast growth factor (FGF) regulates the proliferation of chondrocytes depending on cell adhesion between fibronectin and integrin-α5β1 (22). Our data also showed that integrin-β1 inhibited chondrocyte apoptosis. In the study by Cao et al (23), it was demonstrated that integrin-β1-mediated collagen-chondrocyte interactions protect chondrocytes from apoptosis. The results of the study by Lee et al (24) indicated that mechanical stimulation increased osteoblast proliferation, mediating the regulation by integrin-β1 and integrin-β3 signaling. Furthermore, the overexpression of integrin-β1 enhanced the expression levels of aggrecan and type II collagen for the purpose of maintaining the differentiated phenotype of chondrocytes. Most importantly, type II collagen and aggrecan are the major ECM components responsible for maintaining the phenotypic stability of chondrocytes (3-5). Chondrocyte metabolism is closely associated with collagen and proteoglycan synthesis and decomposition. For instance, type II collagen is the major collagen found in cartilage, accounting for 90-95% of total collagen (25). The qualitative and quantitative changes in type II collagen and proteoglycan synthesis are the direct cause of articular cartilage losing its normal biomechanical properties, having a close association with OA (25,26). The results of the study by Bouchet et al (27) demonstrated that the overexpression of integrin-β1 in cartilage cells had an effect on the maintenance of the chondrocytic phenotype.

GIT1, a scaffold protein, is responsible for PLCγ and ERK1/2 signaling induced by angiotensin II and epidermal growth factor (7-10). According to our results from western blot analysis and siRNA depletion, GIT1 was shown to be a downstream molecule of integrin-β1. In other words, it was shown that integrin-β1 regulated the expression of GIT1, but the upregulation of GIT1 had little effect on the expression of integrin-β1. Moreover, our results demonstrated that GIT1 overexpression had an effect similar to that of integrin-β1 overexpression on the promotion of chondrocyte proliferation and differentiation, as well as the suppression of chondrocyte apoptosis. However, to date, there has been little research focusing on GIT1 expression in cartilage cells, although studies have focused on GIT1 expression in other types of cells. For example, in the study by Rui et al (28), it was demonstrated that GIT1 Y321 phosphorylation is required for ERK1/2- and PDGF-dependent VEGF secretion from osteoblasts to promote angiogenesis and bone healing. In their study, Pang et al (29) demonstrated that the depletion of GIT1 markedly inhibited intima formation following carotid ligation, leading to the reduction of vascular smooth muscle cell (VSMC) proliferation and the stimulation of apoptosis. Additionally, GIT1 exerts effects on vascular remodeling through the PLCγ- and ERK1/2-mediated VSMC proliferation, migration and regulation of apoptosis (29). In another study by Pang et al (30), it was revealed that in global GIT1 knockout mice, cardiomyocyte apoptosis and cardiac dysfunction were enhanced, suggesting the role of GIT1 in post-natal cardiac maturation by mitochondrial biogenesis and function regulation. The results of the study by Zhang et al (31) indicated that cell apoptosis was reduced by GIT through the

Figure 4. Chondrocyte apoptosis is affected by integrin-β1. The cells were seeded in 6-well plates and incubated for 24 h, and then integrin-β1 siRNA transfection was carried out using Lipofectamine 2000. TUNEL-DAPI co-staining assay was used to examine cell apoptosis.
In conclusion, as shown by our results, integrin-β1 exerts a significant effect on the proliferation, apoptosis and differentiation of chondrocytes. More specifically, integrin-β1 stimulates chondrocyte proliferation and differentiation, and suppresses chondrocyte apoptosis by increasing the expression of GIT1. GIT1, as a downstream effector of integrin-β1, exerts similar effects on the proliferation, apoptosis and differentiation of chondrocytes. However, the role of integrin-β1 and its underlying mechanisms of action in regulating the proliferation, differentiation and apoptosis of cartilage cells remain elusive. Therefore, further research is required to provide more compelling evidence of the effects of integrin-β1 on chondrocytes.

References