Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1

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Footnotes
1 Abbreviations used are: ATF, activating transcription factor; ERK, extracellular signal-regulated protein kinase; IGF-1, insulin-like growth factor-1; I-κB, inhibitory κB; IL, interleukin; NF-κB, nuclear factor-κB; NO, nitric oxide; PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; SNP, sodium nitroprusside; TUNEL, terminal deoxynucleotidyl transfer-mediated nick end labeling

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SUMMARY

Cartilage development is initiated by the differentiation of mesenchymal cells into chondrocytes. Differentiated chondrocytes in articular cartilage undergo dedifferentiation and apoptosis during arthritis, in which nitric oxide (NO) production plays a critical role. Here, we investigate the roles and mechanisms of action of insulin-like growth factor-1 (IGF-1) in the chondrogenesis of mesenchymal cells, and maintenance and survival of differentiated articular chondrocytes. IGF-1 induced chondrogenesis of limb bud mesenchymal cells during micromass culture through the activation of phosphatidylinositol (PI) 3-kinase and Akt. PI3-kinase activation is required for the activation of protein kinase C (PKC) α and p38 kinase, and inhibition of extracellular signal-regulated protein kinase (ERK)-1/-2. These events are necessary for chondrogenesis. The growth factor additionally blocked NO-induced dedifferentiation and apoptosis of primary culture articular chondrocytes. NO production in chondrocytes induced downregulation of PI3-kinase and Akt activities, which was blocked by IGF-1 treatment. Stimulation of PI3-kinase by IGF-1 resulted in blockage of NO-induced activation of p38 kinase and ERK-1/-2 and inhibition of PKCα and ζ, which in turn suppressed dedifferentiation and apoptosis. Our results collectively indicate that IGF-1 regulates differentiation, maintenance of the differentiated phenotype and apoptosis of articular chondrocytes via a PI3-kinase pathway that modulates ERK, p38 kinase, and PKC signaling.
INTRODUCTION

Cartilage formation during embryo development is initiated by the differentiation of mesenchymal cells into chondrocytes. Chondrogenesis is triggered by precartilage condensation (i.e., aggregation of mesenchymal cells) that develops into cartilage nodules. Chondrocytes in cartilage either further mature into hypertrophic chondrocytes during endochondral bone formation or remain as permanent chondrocytes throughout their life-cycle in joint articular cartilage. Articular chondrocytes synthesize cartilage-specific extracellular matrix components, such as type II collagen and sulfated proteoglycan, to maintain cartilage homeostasis (1-3). Homeostasis is destroyed during joint cartilage diseases, such as rheumatoid arthritis and osteoarthritis, eventually leading to cartilage destruction, which involves activation of matrix metalloproteinases, loss of differentiated chondrocyte phenotypes (i.e. dedifferentiation) and increase in chondrocyte apoptosis (4, 5).

Differentiation, survival and maintenance of differentiated chondrocyte phenotype are regulated by a number of physical and soluble factors. For example, chondrogenesis of mesenchymal cells during limb bud development is regulated by cell-to-cell adhesion, cell-to-cell matrix interactions and a complicated array of growth factors, including bone morphogenetic protein, fibroblast growth factor, epidermal growth factor, insulin-like growth factor (IGF)-1, parathyroid hormone-related peptide and Indian hedgehog (1). Survival and maintenance of the differentiated phenotype of chondrocytes in articular cartilage is additionally regulated by growth factors and cytokines. For instance, IGF-1 exerts anabolic effects on articular cartilage by stimulating matrix molecule synthesis (6), whereas pro-inflammatory cytokines, such as interleukin (IL)-1, exert catabolic effects during cartilage destruction (7). It is generally believed that effects of pro-inflammatory cytokines are mediated by nitric oxide (NO) production (8). Indeed, direct production of NO in primary culture articular chondrocytes induces dedifferentiation and apoptosis by modulating various intracellular signaling processes, such as activation of p38 kinase and extracellular signal-regulated protein kinase (ERK) (9), inhibition of protein
kinase C (PKC) \(\alpha\) and \(\zeta\) (10), activation of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and accumulation of p53 (11, 12). Chondrogenesis of mesenchymal cells during micromass culture is further regulated by a complicated protein kinase cascade involving protein kinase C (PKC) \(\alpha\) activation, inhibition of ERK, and p38 kinase activation (13-17). Elevated PKC\(\alpha\) and reduced ERK activities are additionally responsible for the maintenance of the differentiated phenotype of articular chondrocytes (18).

Among the anabolic factors for chondrocytes and cartilage, IGF-1 and -2 are single polypeptides homologous to pro-insulin. These growth factors regulate cellular functions by stimulating cell surface receptors, which in turn initiate the phosphorylation cascade (19). IGF-1 is expressed in developing cartilage within the condensing region of the limb as well as in mature cartilage and synovial fluid, where it regulates chondrogenesis of mesenchymal cells and anabolism of cartilage matrix molecules (20). For instance, IGF-1 in synovial fluid enhances proteoglycan synthesis. Elimination of IGF-1 from synovial fluid with neutralizing antibody results in decreased cartilage proteoglycan synthesis and enhanced proteoglycan breakdown (21). In addition, IGF-1 enhances cartilage matrix synthesis, both \textit{in vivo} and \textit{in vitro} (22-24), and exogenous IGF-1 blocks IL-1-induced turnover of proteoglycans in chondrocytes (25, 26). Therefore, IGF-1 appears to function as an inducing signal for chondrogenesis and an anabolic growth factor in cartilage homeostasis. Moreover, this growth factor protects various cell types from apoptosis induced by a variety of stimuli via the activation of complex anti-apoptotic and survival signals, such as phosphatidylinositol 3-kinase (PI3-kinase) and Akt (27, 28). Apoptotic death of articular chondrocytes induced by NO production or Fas ligand is implicated in the pathogenesis of arthritis (29-31). However, it is not known whether IGF-1 prevents apoptosis of primary culture chondrocytes triggered by NO or that of chondrocytes in arthritic cartilage.

IGF-1 has been reported to play a role in the regulation of chondrogenesis of mesenchymal cells and to have anabolic effects on differentiated chondrocytes; however, the signaling mechanisms mediating IGF action are
yet to be elucidated. Moreover, there is no available evidence on IGF-1 regulation of chondrocyte apoptosis. In this study, we evaluate the role of IGF-1 in the regulation of mesenchymal cell chondrogenesis, maintenance of differentiated phenotype and NO-induced apoptosis of knee joint articular chondrocytes. We further investigate the signaling mechanism that mediates IGF-1 action, focusing on the protein kinase cascade. Our data show that IGF-1 induces chondrogenesis of mesenchymal cells and inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes by modulating PI3-kinase, Akt, ERK, p38 kinase and PKC signaling pathways.

**EXPERIMENTAL PROCEDURES**

*Micromass culture of mesenchymal cells* - Mesenchymal cells were isolated from limb buds of Hamburger-Hamilton stage 23/24 chicken embryos and maintained as micromass culture to induce chondrogenesis, as described previously (13, 14). Briefly, cells at a density of $2.0 \times 10^7$ cells/ml in Ham's F12 medium were spotted as 15 µl drops on culture dishes and cultured up to 5 days in the presence of various pharmacological reagents, as stipulated in each experiment. Chondrogenesis was induced by adding the indicated concentrations of IGF-1 in serum-free culture medium, unless otherwise specified. After spotting for 2 h, mesenchymal cells were infected for 2 h with adenoviral control, or adenovirus containing the p85 or p110 subunit of PI3-kinase or wild-type PKCα. Infected cells were cultured up to 5 days in Ham's F12 medium. Chondrogenesis was determined by immunohistochemical analysis of type II collagen expression and accumulation of sulfated proteoglycans using Alcian blue staining, as described previously (14).

*Primary culture of rabbit articular chondrocytes* - Rabbit articular chondrocytes for primary culture were isolated from knee joint cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion for 6 h
with 0.2% collagenase type II (381 units/mg solid, Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) (18). After collecting individual cells by brief centrifugation, cells were resuspended in DMEM supplemented with 10% (v/v) fetal bovine calf serum, 50 µg/ml streptomycin, and 50 units/ml penicillin and plated on culture dishes at a density of 5 × 10^4 cells/cm^2 with medium replacement every 2 days. Cells at day 4 were washed with serum-free medium and treated with the indicated pharmacological reagents, 1 h prior to SNP treatment in the absence of serum. These reagents include IGF-1 (Sigma-Aldrich), LY294002 (Calbiochem), which inhibits PI3-kinase (32), PD98059 (Calbiochem), which inhibits ERK-1/-2 (33), and SB203580 (Calbiochem), a blocker of p38 kinase (34).

**Cartilage explants culture** - Rabbit joint cartilage explants (~125 mm^3) were cultured in serum-free DMEM supplemented with 50 µg/ml streptomycin and 50 units/ml penicillin in the absence or presence of IGF-1 and SNP. For immunohistochemical staining, cartilage explants were fixed in 4% paraformaldehyde for 24 h at 4 °C, dehydrated with graded ethanol, embedded in paraffin, and sectioned into 4 µm slices, as described previously (35). Sections were stained by standard procedures using Alcian blue or antibody against type II collagen (Chemicon, Temecula, CA), and visualized by developing with a kit purchased from DAKO. Apoptotic cells were detected by terminal deoxynucleotidyl transfer-mediated nick end labeling (TUNEL), as described below.

**Apoptosis assay** - DNA fragmentation and TUNEL assays demonstrate that NO-induced death of primary culture articular chondrocytes is due to apoptosis (9). In this study, apoptotic chondrocytes in cartilage explants were detected with a TUNEL assay kit (Roche Diagnostics GmbH), as described previously (36). Briefly, sections of cartilage explants were incubated with a TUNEL mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP. Cells with apoptosis-induced DNA strand breaks were detected by fluorescence microscopy. Alternatively, apoptotic chondrocytes were quantified by flow cytometry by counting
the number of cells displaying fragmented DNA. Briefly, cells were fixed in 80% ethanol and incubated for 1 h with 50 µg/ml propidium iodide, 0.1% Nonidet P-40, and 100 µg/ml RNase A in PBS. The number of cells containing fragmented DNA was quantified on a FACSort flow cytometer with the Cellquest analysis program using approximately $1 \times 10^4$ cells (Becton Dickinson, CA).

**PI3-kinase assay** - Chondrifying mesenchymal cells or primary culture articular chondrocytes were lysed in buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM 4-(2-aminoethyl) benzene-sulfonate fluoride) and phosphotase inhibitors (1 mM sodium orthovanadate and 25 mM NaF). Following removal of cell debris by centrifugation at 4°C for 10 min at 13,000 × g, equal amounts of protein (500 µg) were incubated overnight with antiphosphotyrosine antibody (Transduction Laboratories, Lexington, KY) and collected on protein A-sepharose. Immune complexes were washed three times with PBS containing 1% NP-40, three times with buffer containing 100 mM Tris-Cl (pH 7.5) and 500 mM LiCl, and twice with buffer containing 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. Next, immune complexes were resuspended in 45 µl kinase assay buffer (20 mM Tris-Cl, pH 7.6, 75 mM NaCl, 10 mM MgCl$_2$, 1 mM EGTA), and the kinase reaction was initiated by adding 200 µg/ml phosphatidylinositol and [$\gamma$-$^32$P]ATP (5 µCi/sample) at room temperature for 30 min. Reactions were terminated by adding 100 µl of 1 N HCl. Reaction products were extracted using 200 µl CHCl$_3$:MeOH (1:1) and resolved on a thin layer chromatography silica plate coated with potassium oxalate in solvent containing CHCl$_3$:MeOH:H$_2$O:NH$_4$OH (60:47:11.3:2). The PI3-kinase reaction product (phosphatidylinositol 3-phosphate) was identified by autoradiography.

**Caspase-3 assay** - *In vitro* assay of caspase-3 activity was performed by measuring absorbance of the
cleaved synthetic substrate of caspase-3, Ac-Asp-Glu-Val-Asp-chromophore p-nitroaniline, as described previously (9). Chondrocytes were lysed in the buffer provided in a colorimetric assay kit (Clontech). Lysates (100 µg protein) were reacted with 50 µM Ac-Asp-Glu-Val-Asp-chromophore p-nitroaniline in reaction buffer (0.1 M Hepes, pH 7.4, 20% glycerol, 10 mM dithiothreitol and protease inhibitors, as described above) for 1 h at 37°C and analyzed in an ELISA reader. Enzyme activity was calculated from a standard curve prepared using p-nitroaniline. The relative levels of p-nitroaniline were normalized against the protein concentration of each extract.

PKC and p38 kinase assays - PKCα, PKCζ and p38 kinase activities were determined by immunoprecipitation experiments and an in vitro kinase assay. Briefly, cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and an inhibitor cocktail of proteases and phosphatases, as described above. Cell lysates were incubated with polyclonal antibody against p38 kinase or PKCζ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or monoclonal antibody against PKCα (Transduction Laboratories) for 2 h at 4°C, and immune complexes were precipitated by adding 25 µl protein A-sepharose slurry. The immune complex was resuspended in 20 µl kinase reaction buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl2. The kinase reaction was initiated by adding [γ-32P] ATP (10 µCi/sample) and 1 µg substrate [activating transcription factor-2 (ATF-2) for p38 kinase and myelin basic protein for PKCα and ζ]. After incubation for 30 min at 30°C, the kinase reaction was terminated by adding 4× Laemmli’s sample buffer and boiling for 5 min. Substrate phosphorylation was detected by autoradiography.

NF-κB luciferase assay - NF-κB activity was determined using a reporter gene assay. Briefly, chondrocytes
were either transfected with plasmid containing luciferase and three tandem repeats of serum response element or a control vector. Transfection of the expression vector was performed as described in an earlier report (11). The expression vector (1 µg) was introduced to cells at 30-40% confluency using LipofectAMINE PLUS (Gibco BRL), according to the manufacturer's instructions. Transfected cells were cultured in complete medium for 24 h and luciferase activity determined with an assay kit from Promega. Luciferase activity was normalized against β-galactosidase activity.

Western blot analysis - Whole cell lysates were prepared by extracting proteins in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with an inhibitor cocktail of proteases and phosphatases, as described above. Proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following primary antibodies were employed to detect proteins: mouse anti-chicken type II collagen monoclonal antibody (Chemicon, Temecula, CA), mouse anti-human p53 monoclonal antibody (Santa Cruz Biotechnology Inc.), rabbit anti-human phospho-ERK polyclonal antibody (New England Biolabs, Beverly, MA), mouse anti-human PKCα and PKCζ monoclonal antibody (Transduction Laboratories), rabbit polyclonal antibodies against mouse p38 kinase, human IκBα, human Bax and human active caspase-3 (Santa Cruz Biotechnology Inc.). Blots were developed using a peroxidase-conjugated secondary antibody (Sigma-Aldrich) and an ECL system (Amersham Biosciences).

RESULTS

IGF-1 induces chondrogenesis of mesenchymal cells via a PI3-kinase pathway - Chick limb bud mesenchymal cells were maintained as micromass culture to induce chondrogenesis. Chondrogenic
differentiation was confirmed by examining the expression of type II collagen (a hallmark of chondrocytes) or staining the sulfated proteoglycan with Alcian blue. Mesenchymal cells cultured for 4 days in the presence of 100 ng/ml IGF-1 in serum-free Ham’s F-12 medium synthesized sulfated proteoglycan and expressed type II collagen, as revealed by Alcian blue staining and immunocytochemistry (Fig. 1A), indicating chondrogenic differentiation. An in vitro kinase assay (Fig. 1B) showed that in the absence of serum, IGF-1 activated PI3-kinase and its downstream signaling molecule, Akt (demonstrated by increased phosphorylation) (Fig. 1 C, upper panel). IGF-1 effects on Akt activation were dose-dependent (Fig. 1C, middle panel). Akt phosphorylation was blocked upon treatment with 10 µM LY294002, the PI3-kinase inhibitor (Fig. 1C, lower panel). LY29002 abolished IGF-1-induced accumulation of proteoglycans (Fig. 1D), implying that the growth factor induces chondrogenesis of mesenchymal cells via a PI3-kinase/Akt pathway.

The role of PI3-kinase in serum-induced chondrogenesis was determined by culturing mesenchymal cells with 10% serum in the absence of exogenous IGF-1. PI3-kinase activity was increased during serum-induced chondrogenesis (Figs. 2A and 2B), and LY294002 blocked type II collagen expression (Fig. 2C) and proteoglycan synthesis (Fig. 2D). The role of PI3-kinase in serum-induced chondrogenesis was further confirmed by infecting mesenchymal cells with control adenovirus, or adenovirus encoding a dominant-negative mutant (p85) or constitutively active mutant (p110) of PI3 kinase. As shown in Fig. 2E, expression of p85 was accompanied by a decrease in type II collagen levels, whereas the constitutively active p110 subunit increased type II collagen expression. The above results collectively indicate that chondrogenesis of mesenchymal cells induced by either IGF-1 or serum is dependent on PI3-kinase activation.

IGF-1 induces chondrogenesis by modulating PKC, ERK, and p38 kinase signaling via PI3-kinase - We previously showed that serum-induced chondrogenesis requires increased expression and activity of PKCα, which is necessary for ERK inhibition (13). Additionally, PKCα-independent activation of p38 kinase is a
prerequisite for chondrogenesis (14). Accordingly, we examined whether IGF-1 induces chondrogenesis via a PI3-kinase pathway by modulating PKC\(\alpha\), ERK and/or p38 kinase. PKC\(\alpha\) expression and activity were increased during IGF-1-induced chondrogenic differentiation of mesenchymal cells (Fig. 3A, upper and middle panels). The stimulatory effects of IGF-1 on PKC\(\alpha\) were suppressed by the PI3-kinase blocker, LY294002 (Fig. 3A, lower panel), confirming that the growth factor regulates PKC\(\alpha\) via a PI3-kinase pathway.

PKC\(\alpha\) downregulation with phorbol ester (Fig. 3B) or inhibition with GF109203X (data not shown) resulted in blockage of IGF-1-induced chondrogenesis. However, PKC\(\alpha\) overexpression by adenovirus infection did not induce chondrogenesis in the absence of IGF-1 nor enhance IGF-1-induced chondrogenesis (Fig. 3B). Therefore, it is likely that expression and activation of PKC\(\alpha\) via IGF-1 signaling is necessary, but not sufficient for the induction of chondrogenesis.

We next examined whether ERK and p38 kinase mediate IGF-1-induced chondrogenesis, based on the finding that these two proteins oppositely regulate chondrogenesis of mesenchymal cells (14). During micromass culture of mesenchymal cells in the absence of IGF-1, ERK-1 phosphorylation levels were high at day 1, and dramatically decreased thereafter. ERK-1 phosphorylation was significantly reduced upon the addition of IGF-1 (Fig. 4A, upper panel). In contrast, p38 kinase activity remained low during control micromass culture, but was significantly increased upon IGF-1 treatment (Fig. 4A, upper panel). ERK inhibition and p38 kinase activation by IGF-1 were dose-dependent (Fig. 4A, middle panel). Moreover, IGF-1 effects were abolished by LY294002 treatment (Fig. 4A, lower panel). The ERK inhibitor, PD98059, potentiated IGF-1-induced chondrogenesis of mesenchymal cells, whereas the p38 kinase inhibitor, SB203580, blocked chondrogenesis (Fig. 4B). The above results are consistent with the hypothesis that IGF-1 induces chondrogenic differentiation of mesenchymal cells by activating p38 kinase and inhibiting ERK-1 via a PI3-kinase pathway.

**IGF-1 inhibits NO-induced de differentiation and apoptosis of articular chondrocytes** - We investigated the
role of IGF-1 in NO-induced dedifferentiation and apoptosis of articular chondrocytes. For this purpose, rabbit articular chondrocytes during primary culture were treated with the NO donor, sodium nitroprusside (SNP), for 20 h, either in the absence or presence of 100 ng/ml IGF-1 added 1 h prior to SNP treatment. Direct production of NO induced dedifferentiation of articular chondrocytes. NO effects were blocked by IGF-1 treatment, as confirmed by the inhibition of the NO-induced decrease of sulfated proteoglycan synthesis (Fig. 5A) and type II collagen expression (Fig. 5B). Chondrocytes treated with IGF-1 alone also displayed enhanced sulfated proteoglycan synthesis and type II collagen expression (Figs. 5A and 5B). In addition, NO production induced apoptosis of articular chondrocytes that was blocked by the addition of 100 ng/ml IGF-1 (Fig. 5C). Our data strongly imply that the growth factor inhibits both NO-induced dedifferentiation and apoptosis. As chondrocyte responses in monolayer culture may differ from those in a three-dimensional natural matrix, the effects of NO and IGF-1 on apoptosis and dedifferentiation of chondrocytes were examined during cartilage explant culture. Immunocytochemistry analyses and TUNEL assay revealed that IGF-1 blocked NO-induced inhibition of type II collagen synthesis and apoptotic cell death, respectively, similar to the effects on chondrocytes cultured on plastic (Fig. 6). Thus, IGF-1 blocks NO-induced dedifferentiation and apoptosis of articular chondrocytes cultured either on plastic or in three-dimensional natural matrix.

*IGF-1 blocks NO-induced dedifferentiation and apoptosis of chondrocytes via a PI3-kinase pathway* - The roles of PI3-kinase and Akt in NO-induced apoptosis and dedifferentiation, and the effects of IGF-1 were examined in articular chondrocytes. NO decreased PI3-kinase activity, as determined by an *in vitro* kinase assay (Fig. 7A, lower panel). The phosphorylation status and reduced total Akt protein levels indicate that NO production additionally inhibits Akt activity (Fig. 7A, upper panel). In the absence of NO, IGF-1 transiently activated both PI3-kinase and Akt without modulation of Akt protein levels (Fig. 7B). The decrease in PI3-kinase and Akt activities caused by NO production was abolished upon treatment of chondrocytes with IGF-1.
The specific roles of PI3-kinase and Akt during IGF-1 modulation of NO-induced dedifferentiation and apoptosis of chondrocytes were examined by inhibiting PI3-kinase with LY294002, prior to NO production and IGF-1 treatment. As shown in Fig. 8A, LY294002 abolished the effects of IGF-1 on phosphorylation and expression of Akt, type II collagen expression (Fig. 8A), proteoglycan synthesis (Fig. 8B), and apoptosis of chondrocytes (Fig. 8C). These results collectively suggest that NO induces downregulation of PI3-kinase and Akt signaling, and that IGF-1 inhibits dedifferentiation and apoptosis by blocking NO-induced inhibition of the PI3-kinase and Akt signaling pathways.

**IGF-1 blocks NO-induced dedifferentiation and apoptosis by modulating p38 kinase, ERK and PKC via a PI3-kinase pathway** - Previous studies by our group demonstrate that NO induces activation of p38 kinase, leading to apoptosis and activation of ERK-1/-2, which in turn causes chondrocyte dedifferentiation (9). NO also inhibits PKCζ as a result of p38 kinase activation and PKCα that is independent of ERK or p38 kinase signaling (10). We therefore investigated IGF-1 modulation of apoptosis and dedifferentiation, focusing on the role of NO-induced activation of ERK-1/-2 and p38 kinase and inhibition of PKCα and ζ. Consistent with previous findings (9), NO triggered ERK-1/-2 and p38 kinase activation, as confirmed by Western blot analysis and an *in vitro* kinase assay respectively (Fig. 9A). IGF-1 treatment prior to NO production blocked NO-induced activation of ERK-1/-2 and p38 kinase. Effects of IGF-1 were abolished by the PI3-kinase inhibitor, LY294002 (Fig. 9A). Direct production of NO additionally led to decreased protein levels and activities of PKCα and ζ. Inhibition of PKCα and ζ by NO was blocked by IGF-1 (Fig. 9B). Moreover, treatment with LY294002 abolished IGF-1 effects on PKCα and ζ (Fig. 9C). The significance of IGF-1 modulation of ERK, p38 kinase, and PKCα and ζ was examined by inhibiting individual kinases in the absence of the growth factor. As shown in Fig. 9D, inhibition of NO-induced ERK activation with PD98059 enhanced apoptosis with inhibitory effects on dedifferentiation, whereas blockage of p38 kinase with SB203580 suppressed apoptosis with stimulatory
effects on dedifferentiation. NO-induced apoptosis and dedifferentiation were additionally blocked upon ectopic expression of PKCα or ζ (Fig. 9D). The above data indicate that blockage of NO-induced ERK-1/2 and p38 kinase activation and PKCα and ζ inhibition by IGF-1 via the PI3-kinase pathway is responsible for IGF-1-induced inhibition of apoptosis and dedifferentiation of articular chondrocytes.

In articular chondrocytes, activation of p38 kinase and inhibition of PKCα and ζ by NO induces activation of NF-κB and caspase-3, and accumulation of p53, resulting in apoptotic cell death (11, 12). We examined the role of IGF-1 and PI3-kinase in apoptotic signaling. Consistent with the inhibition of NO-induced upstream apoptotic signals, such as activation of p38 kinase and inhibition of PKCα and ζ, IGF-1 blocked NO-induced degradation of inhibitory κB (I-κB) (Fig. 10A), activation of NF-κB (Fig. 10B) and caspase-3 (Figs. 10A and 10C), and expression of pro-apoptotic Bax (Fig. 10A). Furthermore, the effects of IGF-1 on apoptotic signaling were blocked by LY294002. The results collectively indicate that IGF-1 blocks NO-induced apoptosis by inhibiting apoptotic signaling through the PI3-kinase pathway.

**DISCUSSION**

IGF-1 regulates the chondrogenesis of mesenchymal cells, cartilage and bone development, and maintenance of cartilage homeostasis (20). However, little is known about the regulatory mechanisms of IGF-1 action, including intracellular signal transduction. Upon binding to cell surface receptors, IGF-1 stimulates various signaling molecules, including the PI3-kinase pathway (19, 27). We therefore investigated signaling mechanisms leading to IGF-1 action, focusing on the role of PI3-kinase and its functional relationship with the protein kinase cascade involved in the regulation of chondrogenesis of mesenchymal cells, dedifferentiation, and apoptosis of articular chondrocytes. We demonstrate here that IGF-1 induces chondrogenic differentiation of
chick limb bud mesenchymal cells by activating PI3-kinase, and the subsequent activation of PKCα and p38 kinase and ERK-1/-2 inhibition. We additionally show that IGF-1 inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes through the activation of PI3-kinase and the subsequent blockage of activation of p38 kinase and ERK-1/-2 and inhibition of PKCα and ζ. We also demonstrated that IGF-1 inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes that is caused by the activation of PI3-kinase and resulting blockage of NO-induced activation of p38 kinase and ERK-1/-2 and inhibition of PKCα and ζ.

**IGF-1 regulation of chondrogenesis** - Development of cartilage, which serves as a template for endochondral bone formation, is initiated by the differentiation of mesenchymal cells to chondrocytes. The process is regulated at multiple steps, including condensation of mesenchymal cells and cartilage nodule formation (1). IGF-1 is expressed in developing chondrocytes and cartilage, and regulates chondrogenesis of mesenchymal cells and cartilage development by modulating the function of apical ectodermal ridge and outgrowth of limb buds (20, 22, 37-39). Our current data clearly indicate that chondrogenesis of mesenchymal cells induced by IGF-1 or serum is mediated by the PI3-kinase pathway. This finding is consistent with reports by Hidaka et al. (40) demonstrating that insulin/IGF-1 stimulation of chondrogenesis of the embryonal carcinoma-derived cell line, ATDC5, is induced by Akt activation via PI3-kinase. However, the downstream signaling pathway of PI3-kinase/Akt remains to be elucidated.

We previously showed that serum-induced chondrogenesis during micromass culture requires increased expression and activation of PKCα. Activated PKCα in turn inhibits ERK-1/-2, which induces chondrogenic differentiation. Activation of p38 kinase independent of PKCα is also required for chondrogenesis (14). We demonstrate that PI3-kinase activation is required for the expression and activation of PKCα, inhibition of ERK-1/-2, and activation of p38 kinase, which are necessary signaling events for IGF-1-induced chondrogenesis. It
remains to be determined whether PI3-kinase regulates PKCα and p38 kinase directly or indirectly by modulating intermediate signaling molecules. PI3-kinase activates PDK-1 which in turn stimulates various kinases by phosphorylation, including Akt, PKC and p70 S6 kinase (41). Since p70 S6 kinase activity is required for PKCα expression during chondrogenesis (15), it is important to determine whether PDK-1 and p70 S6 kinase coordinate the regulation of PKCα expression and activation. It is evident that IGF-1 induces chondrogenesis by activating PI3-kinase, followed by the subsequent activation of PKCα and p38 kinase, and ERK-1/2 inhibition. Our current results indicate that expression and activation of PKCα is required, but not sufficient for chondrogenesis. One possible explanation for this observation is that chondrogenesis requires both PKCα activation and modulation of other molecules (such as activation of p38 kinase). Since PKCα overexpression in the absence of IGF-1 did not activate p38 kinase (data not shown), ectopic expression of PKCα alone may be insufficient to induce chondrogenesis in the absence of IGF-1 or serum.

IGF-1 regulation of dedifferentiation and apoptosis of chondrocytes - IGF-1 is produced in differentiated chondrocytes (20, 39) and functions mainly as an anabolic factor, both in vitro and in vivo. For instance, IGF-1 stimulates synthesis of proteoglycan and type II collagen in chondrocytes (22, 23), enhances matrix synthesis and cartilage formation upon in vivo gene delivery (24), and is possibly involved in the repair of damaged cartilage (42). Earlier studies have shown that IGF-1 inhibits the catabolic effects of IL-1 on proteoglycan synthesis in cartilage explants (25, 26) and suppresses the degradation of cartilage matrix components by reducing matrix metalloproteinase-1 and -8 expression and activity (43, 44). In addition, levels of IGF-1 were enhanced in human osteoarthritic synovial fluid (45, 46) and response of arthritic chondrocytes to IGF-1 was reduced (47, 48), suggesting that the growth factor is involved in the pathophysiology of arthritic disease. Based on the assumption that dedifferentiation and apoptosis of articular chondrocytes by NO production are associated with arthritis (8), we examined the role of IGF-1 in NO-induced dedifferentiation and apoptosis. In
this report, we show for the first time that IGF-1 inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes cultured as a monolayer on plastic or in three-dimensional natural matrix in cartilage explants. We additionally observe that IGF-1 blocks dedifferentiation of articular chondrocytes induced by a serial monolayer culture, retinoic acid, epidermal growth factor and interleukin (data not shown).

Data from the current study suggest that PI3-kinase and Akt are downregulated upon NO production in articular chondrocytes as a pre-requisite for NO-induced apoptosis and dedifferentiation. This conclusion is further supported by the finding that IGF-1 stimulates the PI3-kinase/Akt pathway, and that inhibition of PI3-kinase abolishes IGF-1-induced blockage of apoptosis and dedifferentiation. Earlier data have shown that Akt is proteolytically cleaved in either a caspase-dependent or -independent manner during apoptosis, depending on the experimental conditions (49, 50). We did not determine how NO production induces downregulation of PI3-kinase and Akt in chondrocytes. Although Akt is degraded by caspase activation during apoptosis, and IGF-1 blocks Akt degradation by inhibiting caspase activation (49), our data suggest that downregulation of PI3-kinase and Akt are required for the activation of apoptotic signaling such as p38 kinase. It has been suggested that the Akt survival signal blocks apoptosis, not by inhibiting the apoptotic stimulus, but by postponing apoptosis induction, which allows a greater length of time for DNA repair mechanisms to take effect (51). However, during NO-induced apoptosis in articular chondrocytes, clearly the stimulation of the PI3-kinase/Akt pathway by IGF-1 blocks the inducing signal for apoptosis and dedifferentiation (such as activation of ERK-1/-2 and p38 kinase, and inhibition of PKCα and ζ). Therefore, it is likely that downregulation of PI3-kinase and Akt are permissive signals for dedifferentiation and apoptosis. Moreover, IGF-1 inhibits apoptosis and dedifferentiation by inhibiting the downregulation of this permissive signal.

As demonstrated previously, NO production in chondrocytes induces the activation of ERK-1/-2 that is primarily responsible for dedifferentiation with inhibitory effects on apoptosis, and activation of p38 kinase, an inducing signal for apoptosis with inhibitory effects on dedifferentiation (9). In addition, p38 kinase-dependent
and -independent inhibition of PKCζ and α, respectively, are required for NO-induced apoptosis and dedifferentiation, based on the observations that expression and activity of these proteins are decreased by NO and their ectopic expression leads to abrogation of apoptosis and dedifferentiation (10). Moreover, we recently showed that both activation of p38 kinase and inhibition of PKCα and ζ are required for NF-κB activation and p53 accumulation to initiate apoptosis (12). Therefore, the inhibition of apoptosis by IGF-1 is consistent with its ability to block apoptotic signals (p38 kinase activation, PKCα and ζ inhibition, NF-κB activation, p53 accumulation, and caspase-3 activation). Although the inhibition of NO-induced ERK activation by IGF-1 may stimulate apoptosis, we hypothesize that apoptotic signaling blockage overrides the effects of ERK inhibition on apoptosis. Similarly, we suggest that the suppression of ERK-1/-2 activation and PKCα and ζ inhibition by IGF-1 is responsible for inhibiting dedifferentiation, which in turn overrides the effects of IGF-1-induced inhibition of p38 kinase, a condition that potentiates dedifferentiation. An interesting finding of this study is the differential effects of NO production, IGF-1 treatment, and a combination of these two reagents on the activation of ERK and p38 kinase. NO induced relatively long-term activation of ERK and p38 kinase, which was detectable at 3 h after SNP treatment and remained as active up to 24 h (9). IGF-1 alone triggered a more transient activation of ERK and p38 kinase that peaked at 10 min after treatment and returned to the basal levels after 1 h (data not shown). However, NO-induced persistent activation of ERK and p38 kinase was completely blocked upon treatment with IGF-1 as shown in Fig. 9A. When SNP was added to chondrocytes that were pretreated with IGF-1 for 1 h, IGF-1-induced activities of both p38 kinase and ERK were returned to basal levels (data not shown). In contrast to the transient activation of p38 kinase and ERK, IGF-1 induced prolonged activation (up to 24 h) of PI3-kinase/Akt pathway, which blocks NO-induced down regulation of PI3-kinase and Akt (Fig. 7). Because down regulation of PI3-kinase/Akt pathway is necessary for NO-induced long-term activation of p38 kinase and ERK, it is likely that IGF-1-induced activation of PI3-kinase/Akt pathway blocks NO-induced p38 kinase and ERK activation. Although the underlying mechanism is yet to be more clearly elucidated, we
propose that the observed result may be due to activation of the PI3-kinase/Akt pathway by IGF-1, which in turn blocks NO-induced activation of p38 kinase and ERK.

IGF-1 inhibits the apoptosis of various cell types induced by different extracellular stimuli (27, 28). These inhibitory effects of IGF-1 on apoptosis are either dependent (27, 28) or independent (52-54) of the PI3-kinase/Akt pathway, depending on the experimental system. PI3-kinase/Akt inhibits apoptosis by modulating C-Jun N-terminal kinase (55), p53 (56), p21 (57) and Bcl-2 family proteins (58, 59). Our data indicate that in articular chondrocytes, activation of the PI3-kinase/Akt pathway by IGF-1 inhibits NO-induced apoptosis via blockage of pro-apoptotic signaling activation, including p38 kinase and the downstream signaling molecules, NF-κB, p53, Bax and caspase-3.

In summary, we have characterized a number of signaling mechanisms leading to chondrogenic differentiation of chick limb bud mesenchymal cells, blockage of NO-induced dedifferentiation and apoptosis of primary culture rabbit articular chondrocytes. Our results indicate that IGF-1 induces chondrogenesis by activating PI3-kinase, which in turn leads to PKCα activation, inhibition of ERK, and activation of p38 kinase, which are necessary signaling events for chondrogenesis. IGF-1 additionally inhibits NO-induced dedifferentiation and apoptosis through activation of the PI3-kinase/Akt pathway and the subsequent inhibition of NO-induced activation of p38 kinase and ERK and inhibition of PKCα and ζ.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. IGF-1 induces chondrogenesis of mesenchymal cells via the PI3-kinase pathway. A, Mesenchymal cells were maintained as micromass culture for 4 days in the absence or presence of 100 ng/ml IGF-1 in serum-free medium. Accumulated sulfated proteoglycans were stained with Alcian blue, while type II collagen was stained using immunocytochemistry. B, Mesenchymal cells during micromass culture were treated for indicated time-periods with either the vehicle alone as control or 100 ng/ml IGF-1. PI3-kinase activity was determined by measuring phosphorylation of the substrate, phosphatidylinositol 3-phosphate (PIP). C, Mesenchymal cells were cultured for indicated time-periods in the absence (Control) or presence of 100 ng/ml IGF-1 (upper panel), indicated concentrations of IGF-1 for 3 days (middle panel), or 100 ng/ml IGF-1 and indicated concentrations of LY294002 for 3 days (lower panel). Levels of total and phosphorylated Akt were determined by Western blotting. D, Mesenchymal cells were cultured for 4 days in the absence (Control) or presence of 100 ng/ml IGF-1 with or without 10 µM LY294002, and stained with Alcian blue. Data from a typical experiment are presented. Experiments were performed more than five times, with similar results.

Figure 2. PI3-kinase regulates serum-induced chondrogenesis of mesenchymal cells. A and B, Mesenchymal cells were maintained as micromass culture in 10% serum-containing medium for the indicated time-periods in the absence or presence of 10 µM LY294002. PI3-kinase activity was determined using an in vitro kinase assay (A) and type II collagen (Coll-II) was detected by Western blotting (B). C and D, Mesenchymal cells were cultured in serum-containing medium for 4 days in the presence of the indicated concentrations of LY294002. Expression of type II collagen (Coll-II) (C) and accumulation of sulfated proteoglycan (D) were determined by Western blotting and Alcian blue staining, respectively. ERK-1 was detected as a loading control. E, Mesenchymal cells during micromass culture in serum-containing medium were treated for 4 days without (Control) or with 10 µM LY294002 (LY294002), or infected with adenovirus.
containing the p85 regulatory (p85) or p110 catalytic subunits (p110). Expression of type II collagen was determined by immunostaining. Data from a typical experiment are presented. Experiments were performed more than five times, with similar results.

Figure 3. Activation of PI3-kinase by IGF-1 is necessary for PKC\(\alpha\) expression. A, Mesenchymal cells were maintained as micromass culture in serum-free medium for the indicated time-periods in the absence (Control) or presence of 100 ng/ml IGF-1 (upper panel), or in the presence of indicated concentrations (middle panel) or 100 ng/ml (lower panel) of IGF-1 for 3 days without (middle panel) or with (lower panel) the indicated concentrations of LY294002. Expression and activity of PKC\(\alpha\) were determined by Western blotting and an \textit{in vitro} kinase assay using MBP as a substrate, respectively. B, Mesenchymal cells during micromass culture in serum-free medium were treated for 4 days without (Control) or with 100 ng/ml IGF-1 in the absence (IGF-1) or presence of 20 nM phorbol 12-myristate 13-acetate (IGF-1/PMA). Alternatively, cells were transfected with adenovirus encoding PKC\(\alpha\), and then left untreated (PKC\(\alpha\)) or treated with IGF-1 (IGF-1/PKC\(\alpha\)). Accumulation of sulfated proteoglycan was examined by Alcian blue staining. The insert indicates expression levels of PKC\(\alpha\). Data from a typical experiment are presented (n=5).

Figure 4. IGF-1 inhibits ERK and activates p38 kinase via the PI3-kinase pathway. A, Mesenchymal cells were maintained as micromass culture in serum-free medium for the indicated time-periods in the absence (Control) or presence of 100 ng/ml IGF-1 (upper panel) or in the presence of indicated concentrations (middle panel) or 100 ng/ml (lower panel) of IGF-1 and the indicated concentrations of LY294002 for 3 days (middle and lower panels). ERK activation was determined by examining phosphorylation levels using Western blotting (pERK), while p38 kinase activity was determined using an \textit{in vitro} kinase assay with ATF-2 as a substrate (pATF-2). B, Mesenchymal cells during micromass culture were treated for 4 days without (Control) or with
100 ng/ml IGF-1 (IGF-1) in the absence or presence of 10 µM PD98059 (IGF-1/PD) or 10 µM SB203580 (IGF-1/SB). Accumulated sulfated proteoglycan was stained with Alcian blue at day 4. Results of a typical experiment are presented. More than five independent experiments were performed, with similar results.

Figure 5. IGF-1 inhibits NO-induced dedifferentiation and apoptosis of primary culture articular chondrocytes. Primary culture articular chondrocytes were treated with the indicated concentrations of SNP for 20 h in the absence or presence of 100 ng/ml IGF-1. Accumulation of sulfated glycosaminoglycans was quantified by Alcian blue staining (A). Expression of type II collagen was determined by Western blotting, and ERK was detected as a loading control (B). Apoptotic cells were quantified by flow cytometry (C). Data in B represent a typical experiment conducted four times. A and C represent mean values with standard deviation form five independent experiments. The effects of IGF-1 were statistically significant by P value of p<0.05 (*) and p<0.01 (**) 

Figure 6. IGF-1 inhibits NO-induced dedifferentiation and apoptosis of articular chondrocytes during cartilage explant culture. Cartilage explants were left untreated (Control) or treated with 1 mM SNP for 72 h in the absence (SNP) or presence of 100 ng/ml IGF-1 (IGF-1/SNP) or 100 ng/ml IGF-1 with 10 µM LY294002 (LY/IGF-1/SNP). Type II collagen was stained by immunohistochemistry (upper panel) and apoptotic cells were detected with the TUNEL assay (lower panel). Results of a typical experiment are presented.

Figure 7. NO production downregulates PI3-kinase and Akt activities in articular chondrocytes, and IGF-1 blocks the NO effects. Chondrocytes were treated for the indicated time periods with 1 mM SNP (A) or 100 ng/ml IGF-1 (B). Alternatively, chondrocytes were left untreated (-) or treated (+) with 1 mM SNP for 20 h in the presence of the indicated concentrations of IGF-1 (C). Levels of phosphorylated Akt (pAkt) or total Akt
(Akt) were detected by Western blotting (upper panels). PI3-kinase activity was determined using an *in vitro* kinase assay (lower panels). Results of a typical experiment are presented. Experiments were conducted at least four times, with similar results.

**Figure 8. IGF-1 inhibits NO-induced dedifferentiation and apoptosis of primary culture articular chondrocytes via the PI3-kinase pathway.** Chondrocytes were left untreated (-) or treated (+) with 1 mM SNP for 20 h in the absence (-) or presence (+) of 100 ng/ml IGF-1, and indicated concentrations of LY294002 (LY). Levels of phosphorylated Akt (pAkt), total Akt (Akt), and type II collagen (Coll-II) were determined by Western blotting. ERK was detected as a loading control (A). Accumulation of sulfated proteoglycan (B) and apoptotic cells (C) was quantified by Alcian blue staining and flow cytometry, respectively (C). The data in A represent a typical experiment and B and C signify mean values with standard deviation (n = 5).

**Figure 9. IGF-1 blocks NO-induced activation of ERK-1/-2 and p38 kinase, and inhibition of PKCα and PKCζ via the PI3-kinase pathway.** A-C, Chondrocytes were left untreated (-) or treated with (+) 1 mM SNP for 6 h in the presence of the indicated concentrations of IGF-1 and LY294002. Phosphorylated ERK-1 and -2 were detected by Western blotting, while p38 kinase activity was determined using an *in vitro* kinase assay with ATF-2 as a substrate (A). Expression and activity of PKCα and PKCζ were determined by Western blot analysis (WB) and *in vitro* kinase assay, using MBP as a substrate (pMBP) (B and C). D, Proteoglycan synthesis was quantified by Alcian blue staining and expressed as a percentage of the control. Apoptotic cells quantified as a percentage of total cells. Results are presented as data from a typical experiment or mean values with standard deviation. At least four independent experiments were conducted.

**Figure 10. IGF-1 blocks apoptotic signaling via the PI3-kinase pathway in primary culture articular...**
Chondrocytes. A, Chondrocytes were left untreated (-) or treated (+) with 1 mM SNP for 20 h in the absence (-) or presence (+) of 100 ng/ml IGF-1 and 10 µM LY294002. Levels of IκB, p53, active caspase-3, Bax and ERK-1 were determined by Western blotting. B and C, Chondrocytes were left untreated (-) or treated (+) with 1 mM SNP for 20 h with the indicated concentrations (ng/ml) of IGF-1 and 10 µM LY294002. NF-κB activity was determined by a reporter gene assay (B), while caspase-3 activity was determined using a colorimetric assay kit (C). Results are presented as data from a typical experiment (A) or mean values with standard deviation (B and C) (n = 4).
Fig. 1, Oh et al.
Fig. 3, Oh et al.
Alcian blue

B

IGF-1/PD IGF-1/SB

Control IGF-I

pERK

pATF-2

Day

Control       IGF-1

pERK

pATF-2

0  50 100 200

0  0     5 10 LY294002 (µM)

Fig. 4, Oh et al.
Fig. 5, Oh et al.

**A**

Absorbance (% of total)

0 0.5 1 1.5 SNP (mM)

**B**

Collagen (II)

ERK-1

ERK-2

**C**

Apoptosis (% of total)

0 0.5 1 1.5 SNP (mM)
Fig. 6, Oh et al.
Fig. 7, Oh et al.

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Fig. 8, Oh et al.

A

- + + + + SNP
- - + + + IGF-1
0 0 0 10 20 LY (µM)

B

C

Absorbance (% of total)

Apoptosis (% of total)

0 0 0 10 20 LY (µM)
Fig. 9, Oh et al.
Fig. 10, Oh et al.
Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1
Chun-Do Oh and Jang-Soo Chun

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