Maintenance of Differentiated Phenotype of Articular Chondrocytes by Protein Kinase C and Extracellular Signal-regulated Protein Kinase*

Young-Mee Yoon‡§, Song-Ja Kim‡§, Chun-Do Oh‡§, Jung-Won Ju‡, Woo Keun Song§, Yung Joon Yoo‡, Jae-Lin Huh‡, and Jang-Soo Chun‡**

From the ‡National Research Laboratory, Department of Life Science, Kwangju Institute of Science and Technology, Kwangju 500-712, Korea and TG Biotech Co. Ltd., Kyungpook National University, Taegu 702-701, Korea

The differentiated phenotype of chondrocyte is rapidly lost during in vitro culture by a process designated “dedifferentiation.” In this study, we investigate the roles of protein kinase C (PKC) and extracellular signal-regulated protein kinase (ERK) in the maintenance of the differentiated chondrocyte phenotype. Chondrocytes isolated from rabbit articular cartilage underwent dedifferentiation upon serial monolayer culture with cessation of type II collagen expression and proteoglycan synthesis, which was reversed by culturing dedifferentiated cells in alginate gel. The expression pattern of PKCc was essentially the same as that of type II collagen during de- and redifferentiation, in that expression was decreased during dedifferentiation and increased during redifferentiation. In contrast to PKCc, ERK activity increased 15-fold during dedifferentiation. This enhanced activity was terminated during redifferentiation. Down-regulation of PKCc in passage 0 chondrocytes resulted in dedifferentiation. However, overexpression of PKCc did not affect type II collagen levels, suggesting that PKCc expression is not sufficient to maintain the differentiated phenotype. However, inhibition of ERK by PD98059 enhanced type II collagen expression and proteoglycan synthesis in passage 0 cells, retarded dedifferentiation during monolayer cultures, and reversed dedifferentiation caused by down-regulation of PKC. Unlike PKC-dependent ERK regulation of chondrogenesis, PKC and ERK independently modulated chondrocyte dedifferentiation, as confirmed by observations that PKC down-regulation and ERK inhibition did not alter ERK phosphorylation and PKC expression, respectively. In addition, expression of N-cadherin, α-catenin, and β-catenin, which are oppositely regulated to type II collagen during phenotype alterations, were modulated by PKC and ERK during chondrogenesis but not dedifferentiation, supporting distinct mechanisms for the regulation of chondrocyte differentiation and maintenance of differentiated phenotype by these two protein kinases.

Chondrocytes are differentiated from mesenchymal cells during embryo development (1, 2). The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix (ECM) molecules, including type II collagen and proteoglycans such as aggrecan (3–5). The biosynthetic property of chondrocytes is maintained during complex biological processes, including cartilage development, differentiation, and repair. However, the differentiated chondrocyte phenotype is unstable in culture and, therefore, rapidly lost during serial monolayer culture (5–9). This process, designated “dedifferentiation” is a major restriction in mass cell production for cell therapy or tissue engineering of destructive cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocyte morphology transforms into flattened fibroblast-like cells with profound changes in biochemical and genetic characteristics. Dedifferentiation of chondrocytes involves a gradual shift from the synthesis of type II to types I and III collagen. Interestingly, dedifferentiated chondrocytes that do not synthesize cartilage proteins in monolayer culture reexpress the chondrocyte differentiation phenotype when cultured three dimensionally in gels of agarose (10), collagen (11), or alginate (4, 9, 12–14).

Although these alterations in chondrocyte phenotype are well documented, the molecular signal transduction mechanisms involved in this process are yet to be clearly elucidated. We previously showed that chondrogenic differentiation of chick limb bud mesenchymal cells is regulated by complex protein kinase signaling cascades involving protein kinase C (PKC) (15, 16), ERK-1 (16, 17), p38 mitogen-activated protein (MAP) kinase (17, 18), and protein kinase A (19). PKC appears to positively regulate chondrogenesis of mesenchymal cells (15, 16). The PKC multigene family comprises 11 known isoforms (20). Multiple PKC isoforms such as α, ε, ζ, and λ are expressed in differentiating chick limb mesenchymal cells (16). Of these, PKCc exhibits the most marked expression and activation during chondrogenesis. Since the selective inhibition or down-regulation of PKCc is sufficient to block differentiation of mesenchymal cells (16, 18, 19), increased expression of this protein appears to be pivotal in the chondrogenesis process. PKC regulates expression of cell adhesion molecules, such as N-cadherin, fibronectin, and its receptor α5β1 integrin, and hence controls progression of precartilage condensation to cartilage nodules (16).

* This work was supported in part by Korea Research Foundation Grant KRF-2000-015-DP0387 and National Research Laboratory Program (M10104000064). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported in part by the Brain Korea 21 program.
§ Supported by grants from the Korean Ministry of Science and Technology (Life Phenomena and Function Research Group).
¶ To whom correspondence should be addressed: Dept. of Life Science, Kwangju Inst. of Science and Technology, Pook-Gu, Kwangju, 500-712, Korea. Tel.: 82-62-970-2497; Fax: 82-62-970-2484; E-mail: jschun@kjist.ac.kr.

** To whom correspondence should be addressed: Dept. of Life Science, Kwangju Inst. of Science and Technology, Pook-Gu, Kwangju, 500-712, Korea. Tel.: 82-62-970-2497; Fax: 82-62-970-2484; E-mail: jschun@kjist.ac.kr.

1 The abbreviations used are: ECM, extracellular matrix; PKC, protein kinase C; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; P, passage; PMA, phorbol 12-myristate 13-acetate.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
PKC-dependent regulation of chondrogenesis, including the expression of cell adhesion molecules, is exerted via MAP kinase subtype ERK-1 signaling (16, 17). Phosphorylation of ERK-1 (a major subtype of ERK in differentiating mesenchymal cells) was decreased during chondrogenesis, and inhibition of ERK-1 phosphorylation by PD98059 treatment enhanced chondrogenesis up to 2-fold (16). The pattern of ERK-1 phosphorylation was inversely related to the expression and activity of PKCα. Increased expression and activation of PKC was required for the down-regulation of ERK-1 activity, which correlated with induced chondrogenic differentiation of mesenchymal cells. In addition, inhibition or down-regulation of PKC (conditions that inhibit chondrogenesis) resulted in the activation of ERK-1, while inhibition of ERK-1 with PD98059 blocked the inhibitory effects of PKC down-regulation on chondrogenesis (16).

Although the regulation of chondrocyte differentiation by the PKCα-dependent ERK-1 signaling pathway is clearly elucidated, the functions of PKCα and ERK-1 signaling in the maintenance of chondrocyte phenotype have not been examined in detail. The present study investigates the roles of PKCα and ERK-1/2 in the regulation of de- and redifferentiation of chondrocytes and compares these findings with the effects of these proteins on chondrocyte differentiation. We employ serial monolayer culture of chondrocytes derived from rabbit articular chondrocytes, fibroblast-like chondrocytes that were passaged as monolayer, and chondrocytes that had been cultured in alginate gel beads were utilized. Type II collagen was detected using antibodies purchased from Chemicon (Temecula, CA) from whole cell lysates that were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Alternatively, type II collagen expression was determined by Northern blot analysis as described below.

**EXPERIMENTAL PROCEDURES**

**Micromass Culture and Chondrogenesis of Mesenchymal Cells—** Mesenchymal cells were derived from the distal tips of Hamburger-Hamilton stage 23/24 chicken embryo wing buds and maintained as micromass culture to induce chondrogenic differentiation, as described previously (17, 18). Briefly, cells were resuspended in Dulbecco's modified Eagle's medium, and single cells were obtained by collecting the supernatant after brief centrifugation. The cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin (Life Technologies) and then maintained in Ham's F-12 medium containing 10% fetal calf serum and cultured into dish cultures as 15-μl drops. The cells were incubated for 2 h at 37 °C to allow sheet formation and then maintained in Ham's F-12 medium containing 10% fetal calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin either in the absence or presence of various pharmacological reagents, as described for each experiment. Chondrogenesis was determined by examining the expression of cell-associated type II collagen by Western blot analysis and quantified by staining sulfated glycosaminoglycan with alcian blue, as described previously (17, 18).

**Isolation and Monolayer Culture of Rabbit Articular Chondrocytes—** Rabbit articular chondrocytes were released from cartilage slices of 2-week-old New Zealand White rabbits by enzymatic digestion. Briefly, after aseptic dissection cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/mg solid) (Sigma) in Dulbecco's modified Eagle's medium, and single cells were obtained by collecting the supernatant after brief centrifugation. The cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin (Life Technologies) and were then plated on culture dishes at a density of 2 × 10⁴ cells/ml in Ham's F-12 medium containing 10% fetal calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin either in the absence or presence of various pharmacological reagents, as described for each experiment. Chondrogenesis was determined by examining the expression of cell-associated type II collagen by Western blot analysis and quantified by staining sulfated glycosaminoglycan with alcian blue, as described previously (17, 18).

**Cell Culture in Alginite Gel Beads and Recovery of Cells—** Isolated chondrocytes were cultured up to P6, and the fibroblastic cells were cultured in alginate gel beads as described originally by Guo et al. (21). Briefly, cells suspended by trypsin treatment were rinsed with washing solution (0.15 M NaCl, 20 mM HEPES, pH 7.4) and resuspended in 1.25% sodium alginate (Sigma) and 100 μg/ml penicillin and 100 μg/ml streptomycin. The alginate gel beads were incubated at P6 by plating cells at a density of 5 × 10⁴ cells/cm².

**Cell Culture in Alginite Gel Beads and Recovery of Cells—** Isolated chondrocytes were cultured up to P6, and the fibroblastic cells were cultured in alginate gel beads as described originally by Guo et al. (21). Briefly, cells suspended by trypsin treatment were rinsed with washing solution (0.15 M NaCl, 20 mM HEPES, pH 7.4) and resuspended in 1.25% sodium alginate (Sigma) and 100 μg/ml penicillin and 100 μg/ml streptomycin. The alginate gel beads were incubated at P6 by plating cells at a density of 5 × 10⁴ cells/cm².

**Determination of Chondrocyte Phenotype—** Differentiation, dedifferentiation, and redifferentiation of chondrocytes were determined by examining the expression of sulfated glycosaminoglycan by staining with alcian blue or by the expression of type II collagen studied by Western or Northern blot analysis, as described previously (18). Briefly, for Western blot analysis micromass-cultured mesenchymal cells, from freshly isolated chondrocytes, fibroblast-like chondrocytes that were passed as monolayer, and chondrocytes that had been used in alginate gel beads were utilized. Type II collagen was detected using antibodies purchased from Chemicon (Temecula, CA) from whole cell lysates that were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Alternatively, type II collagen expression was determined by Northern blot analysis as described below.

**Northern Blot Assay—** Total RNA was isolated using RNA STAT-60 (TEL-TEST, Inc., Friendswood, TX). For each sample, equivalent amounts of total RNA (15 μg/lane) were denatured and fractionated on formaldehyde/agarose gels. RNA was then transferred to Saran Cyt-N nylon membranes. Prehybridization and hybridization were performed in 250 mM Na₂HPO₄ (pH 7.2), 7% SDS, 1 mM EDTA, 250 mM NaCl, 5% formamide, 50% formamide-sulfate, 50% formamide-sulfate, and 100 μg/ml denatured single strand DNA for 3 and 12 h, respectively. Rabbit type II collagen transcript was probed with partial cDNA generated by reverse transcriptase PCR using RNA isolated from P0 cells. The forward PCR primer for type II collagen was 5'-GGCACCCATGCAGTATCAGTC-3', and the reverse primer was 5'-AGCCCGCATTGATGTCTCC-3'. High-specific activity random-primed probes were prepared from the PCR product (370 bp) using the T7 QuickPrime kit (Amersham Biosciences, Inc.) as specified by the supplier. Filters were washed three times with 0.2×SSC/0.1% SDS and exposed to Kodak X-OMAT film with intensifying screens at −80 °C.

**Western Blot Analysis—** For Western blot analysis, whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS, supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml apropin, and 1 mM 4-(2-aminophenyl) benzensulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄). The proteins were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% nonfat dry milk in Tris-buffered saline. Type II collagen was detected using antibodies purchased from Chemicon (Temecula, CA). PKC isoforms were detected using isoform-specific anti-PKC monoclonal antibodies for α, ε, λ, and ζ (BD Transduction Laboratories, Lexington, KY). Expression of adhesion molecules was determined using antibodies purchased from the following sources: rabbit anti-chick N-cadherin polyclonal antibody from Sigma, rabbit anti-human α-catennin polyclonal antibody from Santa Cruz, and mouse β-catenin monoclonal antibody from BD Transduction Laboratories. The blots were developed using a peroxidase-conjugated secondary antibody and ECL system. Relative abundance of PKCα, pERK, and type II collagen was quantified by densitometric analysis of x-ray film using a GS-710 densitometer and Quantity One program (Bio-Rad, Hercules, CA).

**ERK-1/-2 Assay—** Activation of ERK-1 and-2 was examined by Western blot analysis using antibodies specific to activated, tyrosine- and threonine-phosphorylated ERK-1/2 (New England Biolabs, Beverly, MA), as described previously (17, 18). Proteins were extracted with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, and inhibitors of protease (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml apropin, and 1 mM 4-(2-aminophenyl) benzensulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄) from differentiating mesenchymal cells and dedifferentiating or redifferentiating chondrocytes. Following separation of proteins by electrophoresis, phosphorylation of ERK-1/2 was determined by Western blot analysis.

**Transfection—** To introduce cDNA for wild type PKCα (22) or dominant negative MAP kinase kinase-1 (23), articular chondrocytes either at passage 0 or 2 were transfected with fresh plasmid pEF-BOS/BD and were then processed for Western blot analysis using antibodies specific to PKCα. Transfection of the expression vector was performed as described previously (24). The expression vector (5 μg) was introduced to cells using LipofectAMINE PLUS (Life Technologies) using the procedure recommended by the manufacturer. The transfected cells, which were cultured in complete medium for 48 h, were used for further assays as indicated in each experiment.
Effects of PKC and ERK on Chondrocyte Phenotype

RESULTS

Visualization of PKCa and pERK During Chondrogenesis of Mesenchymal Cells—Micromass culture-induced chondrogenic differentiation of mesenchymal cells was accompanied by increased expression of PKCa and decreased phosphorylation of ERK (Fig. 1A). Our previous biochemical analyses demonstrated that inhibition or down-regulation of PKCa blocked chondrogenesis, whereas inhibition of ERK phosphorylation by PD98059 enhanced this process (16–18). In this study, expression of PKCa and phosphorylation of ERK were visualized in chondrifying mesenchymal cells. Immunocytochemical staining revealed that type II collagen was highly expressed in cells located in cartilage nodules, which are composed of differentiated chondrocytes (Fig. 1B, left panel). Similar to the staining pattern of type II collagen, PKCa staining was strongly positive in cartilage nodule cells at day 4 (Fig. 1B, middle panel). Staining of phosphorylated ERK was strong for the 1-day culture with a homogenous distribution pattern (data not shown), but became very weak in cells at day 4 of culture (Fig. 1B, right panel). This may be due to a reduced amount of phosphorylated ERK at day 4, as shown by Western blot analysis. The remaining phosphorylated ERK was highly stained on the edge of cartilage nodules but completely absent inside of cartilage nodules. Thus, cells synthesizing type II collagen are strongly positive for PKCa staining, but negative for phosphorylated active ERK, supporting our previous conclusion that increased expression of PKCa and decreased ERK activity regulate chondrogenesis of mesenchymal cells.

Changes in PKCa Expression and ERK Phosphorylation during De- and Redifferentiation of Chondrocytes—We initially examined the patterns of PKCa expression and ERK activity during de- and redifferentiation of chondrocytes to determine the roles of these proteins in the maintenance of differentiated chondrocyte phenotype. Rabbit articular chondrocytes were employed to attain a homogeneous differentiated population since cells maintained as micromass culture for 5 days comprise both differentiated chondrocytes and undifferentiated mesenchymal cells. Rabbit articular chondrocytes, seeded into a culture dish at a density of $5 \times 10^4$ cells/cm$^2$, reached confluence by day 4–5. Cells at this stage (designated “P0”) maintained typical chondrocyte morphology, with rounded and polygonal shapes. Serial subculturing of cells to P6 resulted in flattened and fibroblast-like morphology (data not shown).

Type II collagen expression, determined by Northern and Western blot analyses (Fig. 2A), and accumulation of sulfated glycosaminoglycan, established by alcian blue staining (Fig. 2B), were high at P0, began to decrease at P1, and were almost undetectable at P3 and thereafter, confirming typical chondrocyte dedifferentiation by monolayer culture.

PKCa levels decreased during dedifferentiation in an analogous manner to type II collagen expression and accumulation of sulfated proteoglycan (Fig. 2A). The decrease in PKCa expression was observed throughout the culture period, although the degree of reduction was less dramatic than that observed with type II collagen or proteoglycan synthesis (Fig. 2B). Other PKC isoforms expressed in chondrocytes, including ε, ζ, and δ, did not exhibit any significant changes in expression patterns (Fig. 2A). In contrast to the pattern of PKCa expression, low-level ERK-1/2 phosphorylation was observed in primary chondrocytes at P0, and subsequent monolayer culturing resulted in up to 15-fold increase in phosphorylation that was detectable as early as P1. However, no accompanying changes in ERK-1/2 expression were observed (Fig. 2A). Thus, the pattern of ERK-1/2 phosphorylation was reverse to that of type II collagen expression.

Next, we examined changes in PKCa expression and ERK phosphorylation during redifferentiation of dedifferentiated chondrocytes. As shown in Fig. 3A, when cells were cultured three dimensionally in alginate gel beads, the loss of type II collagen expression in monolayer-cultured dedifferentiated chondrocytes was reversed in all examined passages up to P6. Type II collagen expression in P4 cells cultured in alginate gel was detected as early as day 2, and increased as cells were cultured for longer periods (Fig. 3B). Similar to the expression pattern of type II collagen, PKCa expression was decreased in P4 chondrocytes and levels began to increase by day 2 on culturing in alginate gel beads. However, expression of other PKC isoforms remained unaffected on three-dimensional culture (data not shown). In contrast to PKCa, the increased levels of phosphorylated ERK-1/2 in P4 cells in monolayer culture decreased back to concentrations observed in P0 cells in these conditions, but with no corresponding changes in ERK-1/2 expression (Fig. 3B).

The Role of PKCa in the Maintenance of Differentiated Chondrocyte Phenotype—Since the respective decrease and increase in PKCa expression during de- and redifferentiation suggests a possible function in the maintenance of chondrocyte phenotype, the role of this protein was examined by studying inhibition or down-regulation patterns in chondrocytes at P0. For this purpose, primary chondrocytes were treated with PKC inhibitors, GF109203X (25) or Go6976 (26). Inhibition of PKC did not affect type II collagen expression (Fig. 4A) or accumulation of sulfated proteoglycan (data not shown), suggesting that PKC activity blockade is not a signal for inducing dedifferentiation.
Effects of PKC and ERK on Chondrocyte Phenotype

FIG. 2. Changes in PKC expression and ERK phosphorylation during dedifferentiation of chondrocytes. A, rabbit articular chondrocytes were passaged up to P6 as monolayers. Cell-associated type II collagen was detected by Western blotting (WB), and mRNA levels of type II collagen were determined by Northern blotting (NB). PKC isoforms were detected by Western blotting. Phosphorylation of ERK-1/-2 was analyzed by Western blotting with phospho-specific antibody. B, the relative abundance of type II collagen protein and PKCα was quantified by densitometric analyses and expressed as a percentage of P0 cells. Phosphorylation of ERK was expressed as a percentage of maximum phosphorylation, which increased up to 15-fold at P3. Synthesis of sulfated proteoglycan was determined by alcian blue staining and expressed as a percentage of P0 cells. The data in A represent results of a typical experiment conducted eight times, while those in B represent average values with standard deviation by densitometric analyses (n = 8).

Interestingly, however, down-regulation of PKC by prolonged treatment of cells with phorbol 12-myristate 13-acetate (PMA) blocked expression of type II collagen and accumulation of sulfated proteoglycan (Fig. 4B). Since PKC down-regulation by PMA requires initial activation of the protein, cells were treated with PMA in the presence of GF109203X. Under these conditions, PMA treatment led to a 2.5- and 6.4-fold increase in sulfated proteoglycan accumulation in P0 and P2 cells, respectively (Fig. 4D). In addition, expression of dominant negative MAP kinase kinase-1 also rescued expression of type II collagen at P2 (Fig. 6B). Therefore, inhibition of ERK-1/-2 phosphorylation enhanced or restored chondrocyte phenotype (i.e., type II collagen expression and proteoglycan synthesis). ERK in the Maintenance of Differentiated Chondrocyte Phenotype—To investigate the role of ERK-1/-2 in the loss of chondrocyte phenotype during dedifferentiation, the association between ERK-1/-2 phosphorylation and dedifferentiation was investigated. Cells were treated with PD98059 to specifically inhibit ERK-1/-2 (27) at each passage, and expression of chondrocyte markers was examined. Treatment with PD98059 blocked the increased phosphorylation of ERK-1/-2 (Fig. 6A, upper panel) in a dose-dependent manner (Fig. 6C). Inhibition of ERK-1/-2 in P0 cells significantly enhanced type II collagen levels and additionally reversed the inhibition of type II collagen expression in P2 chondrocytes, as determined by Western and Northern blot analyses (Fig. 6A, upper panel). The ability of PD98059 to induce type II collagen expression was proportional to the inhibition of ERK-1/-2 phosphorylation in P2 cells (Fig. 6C). Although the recovery of type II collagen expression by the inhibition of ERK-1/-2 was low at later passages (P4 and P6 cells), ERK-1/-2 inhibition significantly enhanced type II collagen transcript levels during these culture steps (Fig. 6A, lower panel). Consistent with the expression pattern of type II collagen, inhibition of ERK-1/-2 led to a 2.6- and 6.4-fold increase in sulfated proteoglycan accumulation in P0 and P2 cells, respectively (Fig. 6D). In addition, expression of dominant negative MAP kinase kinase-1 also rescued expression of type II collagen at P2 (Fig. 6B). Therefore, inhibition of ERK-1/-2 phosphorylation enhanced or restored chondrocyte phenotype (i.e., type II collagen expression and accumulation of sulfated glycosaminoglycans) in dedifferentiating chondrocytes, signifying that activation of ERK-1/-2 is a signal for inducing dedifferentiation.

In addition to the ability to maintain differentiated chondrocyte phenotype, inhibition of ERK with PD98059 blocked PMA-induced dedifferentiation. Addition of PD98059 to PMA-treated cells reestablished type II collagen expression (Fig. 7A) and proteoglycan synthesis (Fig. 7B). These results suggest that ERK-1/-2 blockage is sufficient to abolish the inhibitory effects of PKC and ERK on Chondrocyte Phenotype

FIG. 3. Changes in PKC expression and ERK phosphorylation during redifferentiation of dedifferentiated chondrocytes. A, rabbit articular chondrocytes were cultured as monolayer (M) to P0 or to the indicated passages (2, 4, and 6). The cells were detached and three dimensionally cultured for four days in alginate gel beads (Alginate). Type II collagen expression was determined by Western blot analysis. B, chondrocytes were maintained as monolayer culture (M) to P0 and P4. P4 cells were detached and cultured in alginate gel beads (Alginate) for indicated periods. Type II collagen expression was determined by Western (WB) and Northern blot (NB) analysis, while ERK-2 and phosphorylated form of ERK-1/-2 (pERK) was examined by Western blotting. The data represent results of a typical experiment, conducted at least six times.
of PKC down-regulation on type II collagen expression in P0 cells. PD98059 did not alter PMA-induced modulation of PKC expression (Fig. 7A) or that of other isoforms (data not shown), implying that the effects of PD98059 are not mediated by regulation of PKC expression. Along with the observation that PMA treatment did not alter ERK-1/-2 phosphorylation, the above results indicate that loss of PKC/H9251 expression and activation of ERK-1/-2 independently regulate de- and redifferentiation of chondrocytes.

The roles of PKC and ERK in dedifferentiation were further characterized by immunostaining of type II collagen, PKC/H9251, and ERK (Fig. 8). P0 chondrocytes were strongly positive for type II collagen staining. However, the fluorescence intensity varied among cells, indicating that P0 chondrocytes consist of a heterogeneous population of cells with different levels of type II collagen expression. The number of type II collagen-expressing cells was dramatically reduced in the P2 phase. Similar to type II collagen, PKC/H9251 levels varied among P0 chondrocytes. However, other isoforms, specifically PKC/H9250, PKC/H9256, and PKC/H9259, were stained homogeneously in P0 cells, and fluorescence intensity remained unchanged during subsequent monolayer cultures (data not shown). Consistent with the results of Western blot analyses, on treatment of P0 chondrocytes with PMA, the staining intensity of type II collagen was dramatically reduced and that of PKC/H9251 was almost undetectable. Immunostaining of phosphorylated ERK-1/-2 was weak in cells at P0; however, a remarkable increase at P2 was observed, which was blocked by the addition of PD98059. Inhibition of ERK-1/-2 by PD98059 in P0 cells resulted in high-level expression of type II collagen and a significant increase in the number of cells highly expressing type II collagen in P2 cells. Addition of PMA and PD98059 did not affect the patterns of phosphorylated ERK and PKC/H9251 expression.

Fig. 4. The role of PKCa in the maintenance of chondrocyte phenotype. A, freshly isolated rabbit articular chondrocytes were seeded into a culture dish at a density of 5 × 10⁴ cells/cm² for 2 days to allow proliferation. The cells were cultured for an additional 48 h in the presence of vehicle alone (C), 1 μM GF109203X (GF), or Go6976 (Go) to inhibit PKC. Expression levels of PKCα, ERK-2, and phosphorylation of ERK were determined by Western blot analyses. B, primary chondrocytes (P0) plated on a culture dish at a density of 5 × 10⁴ cells/cm² for 2 days were treated with the indicated concentrations of PMA for 48 h. Cell-associated type II collagen, PKC isoforms, ERK-2, and ERK phosphorylation patterns were determined by Western blot analyses (upper panel). Levels of PKCs and type II collagen were quantified by densitometric analyses (n = 4). Accumulation of sulfated glycosaminoglycan was determined by alcian blue staining (n = 4). C, P0 chondrocytes cultured for 2 days were treated for 48 h with vehicle alone (Control), or 10 nM PMA (PMA). Alternatively, 1 μM GF109203X was added 30 min prior to PMA treatment (GF + PMA). Cell-associated type II collagen was analyzed by Western blotting. The data in A–C represent results of a typical experiment, which was conducted more than four times.

Fig. 5. Overexpression of PKCa is not sufficient to maintain differentiated phenotype. A, P0 or P2 cells were transfected with empty vector (-) or HA-tagged cDNA for PKCα wild type (+). After 48 h of transfection, expression levels of type II collagen were determined by Western and Northern blot analyses. PKCα, ERK-2, and phosphorylated ERK were detected by Western blotting. B, PKCα and type II collagen were double stained in PKCα-transfected P2 cells using anti-HA and anti-type II collagen antibodies, and photographs were taken with an immunofluorescence microscope. The data in A and B represent results of a typical experiment conducted at least four times.
Differential Regulation of Cell Adhesion Molecule Expression during Modulation of Chondrocyte Phenotype—Unlike PKC-dependent ERK regulation of chondrogenesis (16), the above data indicate that ERK-1/-2 regulates dedifferentiation of chondrocytes in a PKC-independent manner. In an attempt to understand their individual functions, we examined the roles of PKC and ERK in the regulation of cell adhesion molecules, such as N-cadherin, β1-catenin, and β2-catenin, which regulate the differentiation of chondrocytes. Consistent with our previous work (16, 17), chondrogenesis of mesenchymal cells accompanied decreased expression of N-cadherin. Similarly, expression of α- and β-catenin components involved in cadherin-mediated cell-to-cell adhesion decreased during differentiation (Fig. 9A, left panel). The low levels of N-cadherin in differentiated rabbit articular chondrocytes dramatically increased as the differentiated phenotype was lost by serial monolayer culture (Fig. 9A, middle panel). Upon redifferentiation of dedifferentiated cells by alginate gel bead culture, this elevated expression of N-cadherin receded back to levels observed in P0 cells (Fig. 9A, right panel). Treatment with PMA blocked the decrease in N-cadherin expression by day 5 of micromass culture, whereas inhibition of ERK with PD98059 increased N-cadherin expression by day 5 (Fig. 9B). As modulation of PKC and ERK signaling pathways during chondrogenesis altered the expression of cell adhesion molecules (16, 17), the effects of PKC and ERK on the expression of these proteins during de- and redifferentiation of chondrocytes were examined. As shown in Fig. 8A, down-regulation of PKC with PMA blocked the decrease in N-cadherin, α-catenin, and β-catenin expression by day 5 of micromass culture, whereas increased differentiated phenotype by inhibition of ERK with PMA rescued the expression of N-cadherin (Fig. 8B).
PD98059 resulted in further reduction of expression of these molecules (left panel). However, inhibition of PKC or ERK in P0 chondrocytes did not affect expression (Fig. 9B, middle panel), although phenotypes of P0 chondrocytes were lost or enhanced, respectively. Similarly, PKC or ERK inhibition during culturing of dedifferentiated cells in alginate gel did not affect the expression patterns of N-cadherin, α-catenin, and β-catenin (Fig. 9B, right panel). These data collectively indicate that PKC and ERK regulate expression of N-cadherin, α-catenin, and β-catenin during mesenchymal cell differentiation to chondrocytes, but not in de- and redifferentiation of articular chondrocytes.

**DISCUSSION**

Articular chondrocytes, differentiated from mesenchymal cells during embryonic development, are unique among terminally differentiated cells in that they rapidly lose differentiated phenotype upon monolayer culturing on plastic substrata for prolonged periods or repeated passages by a process known as dedifferentiation (3–9). Dedifferentiation is accompanied by profound biochemical changes, including loss of cartilage-specific synthesis of macromolecules, production of interstitial collagens (types I, III, and V), and increase in the synthesis of fibroblast-type proteoglycans (versican) at the expense of aggregan (3–5, 8, 13, 14). As dedifferentiation is a major impediment to mass cell production required for cell therapy or tissue engineering of destructive cartilage, elucidation of the signaling pathway responsible for the gain and loss of cartilage-specific ECM expression should enhance our understanding of the regulatory mechanisms in the maintenance of chondrocyte phenotype. The present study investigates the roles of PKC and ERK in the regulation of chondrocyte phenotype. We demonstrate that the α isoform of PKC is required but not sufficient for the maintenance of differentiated phenotype, whereas activation of ERK-1/-2 is a signal for inducing dedifferentiation, as summarized in Fig. 10. We additionally reveal that PKC and ERK independently regulate dedifferentiation, while chondrocyte differentiation is regulated by PKC-dependent ERK signaling.

Biochemical results from our previous work (16–19) indicate that the expression and activation of PKCα acts as an induction signal for chondrogenic differentiation of mesenchymal cells by micromass culture. For instance, expression of PKCα was low in undifferentiated mesenchymal cells, but dramatically increased before the onset of chondrogenesis in both cytosolic and particulate membrane fractions. Inhibition of PKCα was sufficient to block chondrocyte differentiation. We investigated the role of PKCα in the maintenance of differentiated phenotype by serial monolayer culture of chondrocytes to induce dedifferentiation. In contrast to the expression pattern of PKCα in chondrogenesis, levels of this protein kinase isoform decreased during dedifferentiation, similar to type II collagen. The requirement of PKCα in the maintenance of differentiated phenotype was validated by the observation that down-regulation of the protein in P0 cells caused dedifferentiation. As expected, prolonged treatment of cells with PMA caused down-regulation of phorbol ester-responsive PKCα and ε in chondrocytes. However, since levels of PKCε did not change during dedifferentiation (Fig. 2), it is likely that PMA-induced dedifferentiation is due to loss of PKCα protein. Because PMA-induced down-regulation of PKC requires activation of the protein via conformational changes (28), the PKC inhibitor, GF109203X, was added prior to PMA treatment. Under these conditions, PMA-induced dedifferentiation was completely blocked (Fig. 4C), suggesting that PMA effects are PKC-dependent. Inhibition of PMA-induced dedifferentiation by PKC inhibitors also signifies that PMA effects are not due to the activation of PKC by this compound. Interestingly, treatment of cells with PKC inhibitors (i.e., 1 μM GF109203X or Go6976) did not cause dedifferentiation (Fig. 4A), although high concentrations (i.e., 5 μM) of GF109203X significantly reduced type II collagen expression (data not shown). The finding that GF109203X (1 μM) completely blocked PMA-induced PKC activation (Fig. 4C) indi-
The effects of PKC and ERK on the differentiation and dedifferentiation of chondrocytes. PKC and ERK independently regulate de- and redifferentiation. PKC exerts its effects on differentiation by inhibiting ERK signaling, whereas PKC and ERK independently regulate de- and redifferentiation of chondrocytes.

Cell-to-cell and cell-to-ECM interactions regulate chondrogenesis by coordinating precartilage condensation and cartilage nodule formation. Several cell adhesion molecules such as N-cadherin and integrins are involved in this process. These compounds are additionally involved in chondrocyte survival and differentiation. In an attempt to determine the regulatory mechanisms of PKC and ERK, we examined whether expression patterns of these molecules undergo changes during de- and redifferentiation. We focused on the expression of molecules involved in cell-to-cell adhesion such as N-cadherin, which is generally down-regulated during progression from precartilage condensation to cartilage nodule formation in chondrogenesis. N-cadherin, α-catenin, and β-catenin, which are required for cadherin-mediated cell-to-cell interactions, exhibited reverse expression patterns to that of type II collagen, as shown in Fig. 9. PKC and ERK regulated expression of adhesion molecules during chondrogenesis of mesenchymal cells, but not during de- and redifferentiation. Therefore, unlike the differentiation process, PKC and ERK regulation of de- and redifferentiation are not mediated by cell adhesion molecules.

Since round chondrocytes transform into flattened fibroblast-like cells during dedifferentiation, modification of the actin skeleton is implicated in the de- and redifferentiation of these cells. Indeed, the microfilament-disruptive drug, cytochalasin, induces reexpression of chondrocyte phenotype in monolayers of both subcultured and retinoic acid-modulated chondrocytes. Although microfilament modification is an important mediator of the chondrocyte phenotype, ERK regulation of chondrocyte dedifferentiation is not mediated by the modification of actin cytoskeleton. For instance, although disruption of F-actin by cytochalasin D caused reexpression of type II collagen in P2 and P4 cells, stimulation of type II collagen expression by the inhibition of ERK-1/2 phosphorylation did not lead to microfilament modification, as determined.
Effects of PKC and ERK on Chondrocyte Phenotype

by fluorescence microscopy or reversion of flattened morphology to the round cell shape (data not shown). Thus, the stimulatory effects of ERK-1/2 inhibition were not due to changes in cell morphology or microfilament modification. We are currently investigating the mechanisms of PKC and ERK regulation of chondrocyte de- and redifferentiation.

In summary, we demonstrate that maintenance of the differentiated phenotype of articular chondrocytes is regulated by PKCα and ERK signaling mechanisms, which are also involved in mesenchymal cell differentiation to chondrocytes, as confirmed by observations that loss of PKCα expression or activation of ERK-1/2 causes dedifferentiation of chondrocytes during subcultures. Chondrocyte differentiation is mediated by the PKC-dependent ERK regulation of cell adhesion molecules, while PKCα and ERK separately regulate dedifferentiation of chondrocytes, independent of N-cadherin, α-catenin, or β-catenin expression modulation.

REFERENCES

1. Solursh, M. (1989) Curr. Opin. Cell Biol. 1, 989–994
2. Sandell, L. J., and Adler, P. (1999) Front. Biosci. 4, 741–742
3. Hauselman, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kuettnner, K. E., and Thomar, J. M. A. (1994) J. Cell Sci. 107, 17–27
4. Reginato, A. M., Iozzo, R. V., and Jimenez, S. A. (1994) Arthritis Rheum. 37, 1338–1349
5. Archer, C. W., McDowell, J., Bailey, M. T., Stephens, M. D., and Bentley, G. (1990) J. Cell Sci. 97, 361–371
6. Benya, P. D., Padilla, S. R., and Nimni, M. E. (1977) Biochem. 16, 865–872
7. Benya, P. D., Padilla, S. R., and Nimni, M. E. (1978) Cell 15, 1313–1321
8. Lefebvre, V., Feeters-Doria, C., and Vaes, G. (1990) Biochim. Biophys. Acta 1051, 266–275
9. Bonaventure, J., Kadhim, N., Cohen-Solal, L., Ng, K. H., Bourguignon, J., Lasselin, C., and Freisinger, P. (1994) Exp. Cell Res. 212, 97–104
10. Benya, P. D., and Shaffer, J. D. (1982) Cell 30, 215–224
11. Thenet, S., Benya, P. D., Demignot, S., Feunteun, J., and Adolph, M. (1992) J. Cell. Physiol. 150, 158–167
12. Loty, S., Saunier, J.-M., Loty, C., Boulekbache, H., Kokubo, T., and Forest, N. (1998) J. Biomed. Mater. Res. 42, 213–222
13. Lemare, F., Steinberg, N., Griel, C. L., Demignot, S., and Adolph, M. (1998) J. Cell. Physiol. 176, 303–313
14. Demoor-Fossard, M., Redini, F., Boittin, M., Pujol, J.-P. (1998) Biochim. Bio-
Maintenance of Differentiated Phenotype of Articular Chondrocytes by Protein Kinase C and Extracellular Signal-regulated Protein Kinase

Young-Mee Yoon, Song-Ja Kim, Chun-Do Oh, Jung-Won Ju, Woo Keun Song, Yung Joon Yoo, Tae-Lin Huh and Jang-Soo Chun

J. Biol. Chem. 2002, 277:8412-8420.
doi: 10.1074/jbc.M110608200 originally published online December 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110608200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at http://www.jbc.org/content/277/10/8412.full.html#ref-list-1