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Immunosuppressant rapamycin inhibits protein kinase C α and p38 mitogen-activated protein kinase leading to the inhibition of chondrogenesis

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Abstract

Immunosuppressants are now known to modulate bone metabolism, including bone formation and resorption. Because cartilage, formed by differentiated chondrocytes, serves as a template for endochondral bone formation, we examined the effects of the immunosuppressant rapamycin on the chondrogenesis of mesenchymal cells and on the cell signaling that is required for chondrogenesis, such as protein kinase C, extracellular signal-regulated kinase-1 (ERK-1), and p38 mitogen-activated protein (MAP) kinase pathways. Rapamycin inhibited the expression of type II collagen and the accumulation of sulfate glycosaminoglycan, indicating inhibition of the chondrogenesis of mesenchymal cells. Rapamycin treatment did not affect precartilage condensation, but it prevented cartilage nodule formation. Exposure of chondrifying mesenchymal cells to rapamycin blocked activation of the protein kinase C α and p38 MAP kinase, but had no discernible effect on ERK-1 signaling. Selective inhibition of PKCα or p38 MAP kinase activity, which is dramatically increased during chondrogenesis, with specific inhibitors in the absence of rapamycin blocked the chondrogenic differentiation of mesenchymal cells. Taken together, our data indicate that the immunosuppressant rapamycin inhibits the chondrogenesis of mesenchymal cells at the post-precartilage condensation stage by modulating signaling pathways including those of PKCα and p38 MAP kinase.

Keywords: Chondrogenesis; Mesenchymal cells; Micromass culture; Protein kinase C α; p38 MAP (mitogen-activated protein) kinase; Rapamycin

1. Introduction

Immunosuppressants such as rapamycin and tacrolimus (FK506) are well known to affect a range of essential cellular functions, including progression of the cell cycle and DNA repair and recombination (Brown and Schreiber, 1996; Keith and Schreiber, 1995). A recent study also indicated that immunosuppressants regulate cell differentiation such as adipogenesis (Bell et al., 2000). Rapamycin exerts its effects by forming a stable complex with the cellular target FK506-binding protein. The complex binds to a family of kinases, designated mammalian target of rapamycin (mTOR[1]), which belong to a family of phosphatidylinositol kinase-related kinases (Brown and Schreiber, 1996; Chou and Blenis, 1995). Among the cellular functions affected by immunosuppressant, recent studies indicated that immunosuppressants affect bone metabolism including the formation and resorption of bone. For instance, FK506 caused a marked acceleration of bone remodeling in the tibial metaphysis, leading to a loss of trabecular bone volume (Cvetkovic et al., 1994; Voggnereiter et al., 2000). It was also noted that large quantities of bone were induced when animals were treated with the immunosuppressant cyclosporin A or FK506 (Voggnereiter et al., 2000; Ekelund and Nilsson, 1992). Because cartilage serves as a template for endochondral bone formation and because cartilage development is initiated by the differentiation of mesenchymal cells into chondrocytes (Ahrens et al., 1977; Sandell and Adler, 1999; Solursh, 1989), it is of interest to know whether immunosuppressants affect chondrogenesis.

Micromass culture of dissociated chick limb bud mesenchymal cells is frequently used as a model system to

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study chondrogenesis (Ahrens et al., 1977; Sandell and Adler, 1999). Chondrogenic differentiation of mesenchymal cells is accompanied by morphological changes such as precartilage condensation and cartilage nodule formation. Precartilage condensation is mediated by extensive cell-to-cell and cell-to-extracellular matrix interactions, followed by differentiation into cartilage nodules in which differentiated chondrocytes are surrounded by cartilage-specific extracellular matrix components such as type II collagen and aggrecan (Sandell and Adler, 1999; Solursh, 1989). Several cell adhesion molecules such as N-cadherin (Oberlender and Tuan, 1994; Tavella et al., 1994), integrins (Shakibaei, 1998; Tavella et al., 1997), and ECM components including fibronectin, type I collagen, laminin, and tenascin (Tavella et al., 1997; Maleski and Knudson, 1996; Pacifici et al., 1993) are known to regulate both precartilage condensation and cartilage nodule formation.

Chondrogenesis is regulated by complex protein kinase signaling cascades that include protein kinase C (Sonn and Solursh, 1993; Choi et al., 1995), extracellular signal-regulated kinase (ERK) (Chang et al., 1998), p38 mitogen-activated protein (MAP) kinase (Oh et al., 2000), and protein kinase A (Lee and Chuong, 1997; Yoon et al., 2000a). We previously found that protein kinase C positively regulates the chondrogenesis of mesenchymal cells (Choi et al., 1995). The protein kinase C multigene family consists of 11 known isoforms (Mellor and Parker, 1998). Multiple protein kinase C isoforms such as α, ε, ζ, and λ/ι are expressed in differentiating chick limb mesenchymal cells (Chang et al., 1998). Most evident among the protein kinase C isoforms expressed during chondrogenesis is the increased expression and activation of protein kinase C α. It has been shown that the selective inhibition or down-regulation of protein kinase C α is sufficient to block chondrogenesis (Yoon et al., 2000b), suggesting a pivotal role for protein kinase C α in chondrogenesis. The protein kinase C-dependent regulation of chondrogenesis appears to be exerted via MAP kinase subtype ERK-1 signaling (Chang et al., 1998). The increased expression and activation of protein kinase C are required for down-regulation of ERK-1 activity, which correlates with the induction of chondrogenic differentiation of mesenchymal cells. In contrast to ERK-1, p38 MAP kinase activity is increased during chondrogenesis in a protein kinase C-independent manner, and inhibition of p38 MAP kinase blocks chondrogenesis (Oh et al., 2000). Therefore, the p38 and ERK-1/-2 MAP kinase subtypes regulate chondrogenesis at the post-precartilage condensation stage by acting as positive and negative regulators of chondrogenesis, respectively (Oh et al., 2000).

In the present study, we first examined the effects of the immunosuppressant rapamycin on the chondrogenic differentiation of chick limb bud mesenchymal cells. We also investigated the possible modulation by rapamycin of signaling pathways such as protein kinase C, ERK-1, and p38 MAP kinase pathways that are required for chondrogenesis. Here, we report that rapamycin inhibits the chondrogenic differentiation of mesenchymal cells by blocking the activation of protein kinase C α and p38 MAP kinase during the progression of precartilage condensation to cartilage nodule formation.

2. Materials and methods

2.1. Micromass culture of mesenchymal cells

Mesenchymal cells were derived from the distal tips of Hamburger–Hamilton stage 23/24 chicken embryo limb buds and maintained as micromass cultures to induce chondrogenesis as described previously (Oh et al., 2000; Yoon et al., 2000b). Briefly, the cells were suspended at a density of 2×10^7 cells/ml in Ham's F-12 medium containing 10% fetal calf serum and spotted as a 15-μl drop into culture dishes. The cells were incubated for 2 h at 37 °C to allow attachment 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 in each experiment. Chondrogenesis was determined by examining the expression of type II collagen by immunocytochemistry and quantified by staining sulfated cartilage matrix with Alcian blue.

2.2. Immunocytochemical detection of type II collagen

Chondrifying mesenchymal cells (3 spots/35-mm dish) were fixed with 3% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature. The cells were washed and then incubated for 1 h with anti-collagen type II monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), and type II collagen was visualized using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) following the procedure recommended by the manufacturer.

2.3. Determination of precartilage condensation

Precartilage condensation was verified by staining the cells with peanut agglutinin, which is a specific marker of precartilage condensation (Maleski and Knudson, 1996; Aulthouse and Solursh, 1987). Peanut agglutinin binds cell aggregates or precartilage condensation before the deposition of cartilage-specific extracellular matrix components, which makes it possible to distinguish precartilage condensation from differentiated cartilage nodules. Briefly, differentiating mesenchymal cells (3 spots/35-mm dish) were fixed with 3% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, and the cells were washed and incubated for 1 h with 0.05 mg/ml of biotinylated peanut agglutinin (Vector Laboratories). Peanut agglutinin binding was visualized by color development

using a Vectastain ABC kit (Vector Laboratories), following the procedure recommended by the manufacturer.

2.4. Cell proliferation assay

Proliferation of mesenchymal cells during micromass cultures was determined by directly counting viable cells (Chang et al., 1998; Oh et al., 2000). Mesenchymal cells were maintained as micromass cultures (3 spots/35-mm dish) in the absence or presence of various reagents for the indicated time periods. Then the cells were suspended in a 0.1% solution of trypsin and collagenase, and the number of viable cells was counted in triplicate, using a hemocytometer.

2.5. Protein kinase C assay

Expression of protein kinase C isoforms was determined by Western blot analysis of whole-cell lysates prepared by extracting proteins using buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate and supplemented with protease inhibitors [10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride]. The proteins were size-fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Protein kinase C isoforms were detected with isoform-specific anti-protein kinase C monoclonal antibodies for ε, λ/ι, or ζ (Transduction Laboratories, Lexington, KY, USA) or with polyclonal antibody for α (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were developed using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence system.

The activation of protein kinase C isoforms was determined by examining the translocation of cytosolic protein kinase C to the particulate membrane after cell fractionation as described previously (Oh et al., 2000; Yoon et al., 2000a). Briefly, cells maintained as micromass cultures for the indicated periods of time were scraped off in a buffer containing 20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 2 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 2 mM ethylenediaminetetraacetic acid (EDTA) supplemented with inhibitors of protease and phosphatase (5 mM sodium fluoride and 1 mM sodium orthovanadate). The cells were sonicated twice for 6 s each and then centrifuged at 100,000 × *g* for 1 h. The supernatant was saved as the cytosolic fraction. The pellet was extracted with a buffer containing 20 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors. Following centrifugation at 15,000 × *g* for 15 min, the supernatant was saved as the particulate membrane fraction. The distribution of protein kinase C isoforms was determined by Western blot analysis.

2.6. ERK assay

Activation of ERK-1 was examined by Western blot analysis using antibody specific to activated, tyrosine- and threonine-phosphorylated ERK-1 and -2 (New England Biolabs, Beverly, MA, USA) as described previously (Yoon et al., 2000a,b). Mesenchymal cells were maintained as micromass cultures in the absence or presence of various reagents for the indicated time periods, and proteins were extracted with a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecylsulfate, and inhibitors of protease and phosphatase. Following separation of proteins by electrophoresis, the phosphorylation of ERK-1 was determined by Western blot analysis.

2.7. p38 MAP kinase assay

The activity of p38 MAP kinase was determined by immune complex kinase assay, using activating transcription factor-2 (ATF-2) as substrate and a procedure described previously (Oh et al., 2000; Yoon et al., 2000b). Briefly, cell lysates were prepared in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophophate, and inhibitors of protease and phosphatase. Proteins were precipitated with rabbit polyclonal anti-p38 MAP kinase antibody (Santa Cruz Biotechnology) and protein A-agarose beads (Pierce, Rockford, IL, USA). After being washed with lysis buffer, the beads were resuspended in 50 μl kinase reaction buffer containing 25 mM Tris–HCl, pH 7.5, 5 mM β-glycerophophate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, [^{32}P]γATP, and 2 μg ATF-2 fusion protein as a substrate for p38 MAP kinase (New England Biolabs). Following incubation for 30 min at 30 °C, the reaction was stopped by addition of 4 × Laemmli's sample buffer followed by boiling. Samples were resolved by electrophoresis, and the phosphorylation of activating transcription factor-2 was then determined by autoradiography.

2.8. p70 S6 kinase assay

Activation of p70 S6 kinase was examined by mobility-shift assay in a gel. Whole-cell lysates were prepared by extracting proteins with a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate supplemented with inhibitors of protease and phosphatase as described above. The proteins were separated by electrophoresis through a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. p70 S6 kinase was detected by Western blot analysis, using anti-p70 S6 kinase antibody (Santa Cruz Biotechnology).

2.9. Data analyses and statistics

The results are expressed as the mean ± S.E.M. values from the number of determinations indicated. Student's *t*-test was used for comparing individual treatments with their respective control values. A probability of $P < 0.05$ was accepted as denoting a significant difference.

3. Results

3.1. Rapamycin inhibits micromass culture-induced chondrogenesis of mesenchymal cells

Chick limb bud mesenchymal cells were maintained as micromass cultures to induce chondrogenesis. Chondrogenic differentiation was confirmed by examining the expression of type II collagen, a hallmark of chondrocytes (Fig. 1A). Treatment of mesenchymal cells with the immunosuppressant rapamycin inhibited type II collagen expression (Fig. 1A). Furthermore, Alcian blue staining of sulfated glycosaminoglycan indicated that the inhibitory effects of rapamycin on chondrogenesis were dose dependent (Fig. 1B). Since 1 nM rapamycin reduced chondrogenesis to 17% compared to that in control cells, this dose was used in subsequent experiments unless otherwise indicated.

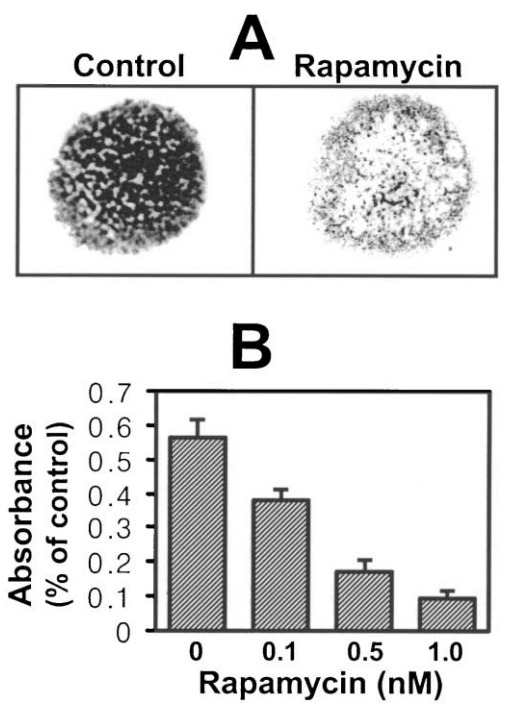

Fig. 1. Rapamycin inhibits chondrogenesis. (A) Mesenchymal cells were maintained as micromass cultures for 4 days in the absence or presence of 1 nM rapamycin, and expression of type II collagen was determined by immunocytochemistry. (B) Chondrogenesis was quantified by staining sulfated glycosaminoglycan with Alcian blue and measuring the absorbance of bound Alcian blue extract at 600 nm. The experiments were done six times. The data in (A) represent the results of a typical experiment and (B) shows means ± S.E.M. Each point in (B) is $P < 0.05$, compared to the control.

3.2. Rapamycin regulates proliferation of chondrifying mesenchymal cells

In an attempt to define the target stage(s) of chondrogenesis regulated by rapamycin, we first asked whether rapamycin had an effect on the proliferation of mesenchymal cells because chondrogenesis depends on cell density. As shown in Fig. 2A, the treatment of mesenchymal cells with rapamycin reduced cell proliferation in a dose-dependent manner. However, the inhibitory effects of rapamycin on cell proliferation were much less dramatic than those on chondrogenesis. For instance, 1 nM rapamycin inhibited cell proliferation and chondrogenesis to 45% and 17% of the control level, respectively. To further test whether the reduced cell proliferation is associated with the inhibition of chondrogenesis, we increased the cell density by twofold, i.e. from 2×10^7 to 4×10^7cells/ml. In comparison to a density of 2×10^7 cells/ml, in cells cultured at a density of 4×10^7 cells/ml there was 218% and 263% increase in chondrogenesis and cell number, respectively (Fig. 2B). Treating cells at 4×10^7 cells/ml density with 1 nM rapamycin resulted a reduced chondrogenesis (28%) and high cell density (186%) when compared to control cells (i.e. cells cultured at 2×10^7 cells/ml without treatment), respectively (Fig. 2B). The above results indicate that rapamycin inhibited chondrogenesis even in the presence of a high cell density. Taken together, our results suggest that it is unlikely that rapamycin inhibited chondrogenesis through the reduction of cell number.

3.3. Rapamycin regulates progression from precartilage condensation to cartilage nodule formation

We next examined whether rapamycin regulated chondrogenesis by modulating precartilage condensation or by affecting cellular events initiated by precartilage condensation. Precartilage condensation was verified by staining the cells with peanut agglutinin which specifically marks precartilage condensation. As shown in Fig. 3, on day 2 of the culture, numerous precartilage condensation were present which had not differentiated into cartilage nodules, as evidenced by the absence of Alcian blue staining. The precartilage condensation grew larger and progressed to form cartilage nodules on day 4 of culture, as indicated by Alcian blue staining. Rapamycin did not significantly affect precartilage condensation on day 2 of culture; however, the coalescence of cellular aggregates and their pro-

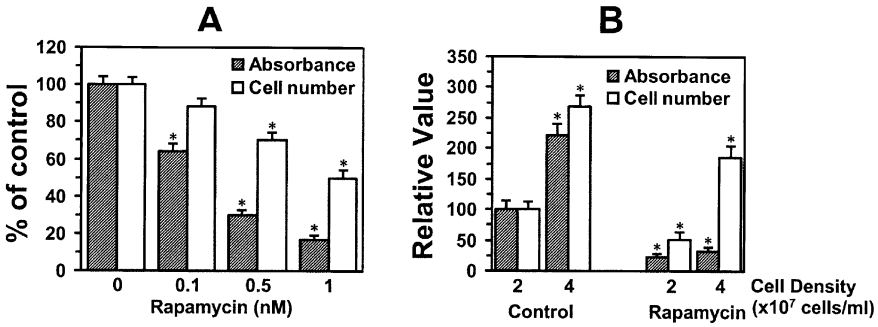

Fig. 2. Rapamycin-induced inhibition of cell proliferation is not responsible for the inhibition of chondrogenesis. (A) Mesenchymal cells were cultured for 4 days in the presence of increasing concentrations of rapamycin. Cell numbers were determined by incubating cells with trypsin, followed by counting with a hemocytometer. Chondrogenesis was quantified by measuring the absorbance of Alcian blue staining. (B) Cells were cultured at densities of 2 or 4×10^7/ml in the absence or presence of 1 nM rapamycin. The number of cells and chondrogenesis were determined after 4 days of culture. The data represent means ± S.E.M. ($n = 6$). $^*P < 0.05$, compared to the control (i.e. cells cultured at 2×10^7 cells/ml without treatment).

gression to cartilage nodules on day 4 of culture was significantly blocked in cells treated with 1 nM rapamycin (Fig. 3). Thus, rapamycin appears to regulate the cellular events involved in the progression from the precartilage condensation stage to cartilage nodule formation.

3.4. Rapamycin inhibits activation of protein kinase C α during chondrogenesis

Because the chondrogenesis of mesenchymal cells, including the progression of precartilage condensation to cartilage nodule formation, is regulated by protein kinase signaling cascades, including protein kinase C, ERK-1, and p38 MAP kinase, we explored the possibility that the inhibitory effect of rapamycin on chondrogenesis might be brought about by modulation of protein kinase signaling cascades. We first examined whether rapamycin effects an increase in the expression of protein kinase C α which is required for chondrogenesis. Consistent with our previous observation (Oh et al., 2000; Yoon et al., 2000b), the expression of protein kinase C α increased as chondrogenesis progressed, whereas the expression of protein kinase C ζ and λ/ι did not vary significantly during chondrogenesis (Fig. 4A). However, when cells were treated with rapamycin, the expression of protein kinase C isoforms was not significantly affected (Fig. 4A), suggesting that the inhibitory effects of rapamycin on chondrogenesis were not caused by the modulation of PKC isoform expression.

Next, we checked whether rapamycin affects the activation of protein kinase C by examining the translocation of cytosolic protein kinase C to the particulate membrane fraction, because protein kinase C activation requires a stable association of cytosolic protein kinase C with membrane phospholipid and diacylglycerol. Among the protein kinase C isoforms, translocation to the particulate membrane fraction during micromass culture was most evident for protein kinase C α. The amount of protein kinase C ε

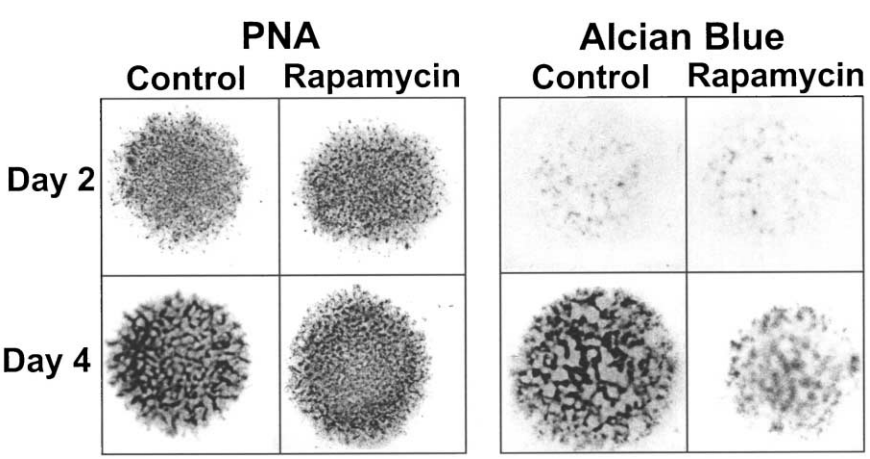

Fig. 3. Rapamycin inhibits progression of precartilage condensation to cartilage nodules. Mesenchymal cells were cultured in the presence of vehicle alone as control or 1 nM rapamycin for 2 or 4 days. The cells were stained with peanut agglutinin (PNA) to determine precartilage condensation or with Alcian blue to determine chondrogenesis. The data represent the results of a typical experiment of at least five independent experiments.

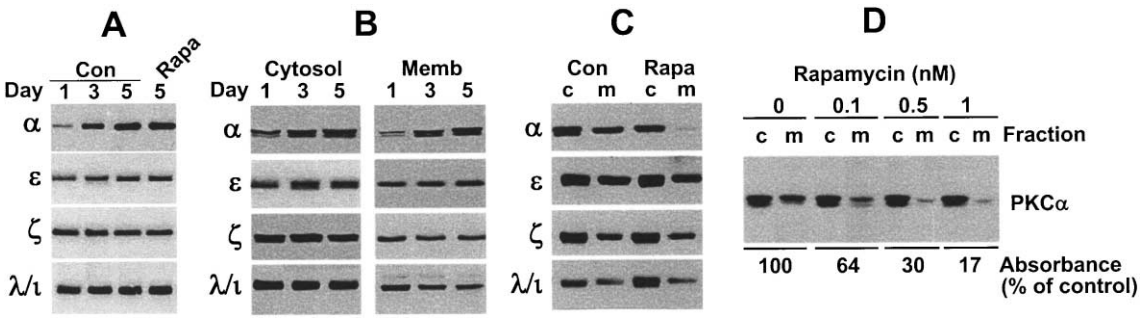

Fig. 4. Rapamycin inhibits activation of PKCα. (A) Mesenchymal cells were maintained as micromass cultures for the indicated time periods in the absence (Con) or presence of 1 nM rapamycin (Rapa), and the expression of PKC isoforms was determined by Western blot analysis. (B) Distribution of PKC isoforms between cytosolic (Cytosol) and particulate membrane (Memb) fractions was determined for cells cultured for the indicated time periods. (C) Distribution of PKC isoforms between cytosolic (c) and particulate membrane (m) fractions was determined for cells cultured for 5 days in the absence (Con) or presence of 1 nM rapamycin (Rapa). (D) Cells were cultured for 5 days in the presence of the indicated concentrations of rapamycin, and translocation of cytosolic protein kinase C (PKC) α to the particulate membrane fraction was examined by Western blot analysis. The data represent the results of a typical experiment conducted at least five times.

in the particulate membrane fraction also increased, albeit slightly, while the levels of protein kinase C ζ and λ/ι in the membrane fraction slightly decreased during chondrogenesis (Fig. 4B). The translocation of cytosolic protein kinase C α to the particulate membrane fraction was dramatically reduced when cells were treated with 1 nM rapamycin, whereas the distribution of the other protein kinase C isoforms was not affected (Fig. 4C). The extent of the inhibitory effects of rapamycin on protein kinase C α translocation was dose dependent and consistent with the degree of inhibition of chondrogenesis (Fig. 4D). Because selective inhibition or down-regulation of protein kinase C α is sufficient to inhibit chondrogenesis (Chang et al., 1998; Yoon et al., 2000b), the above results strongly

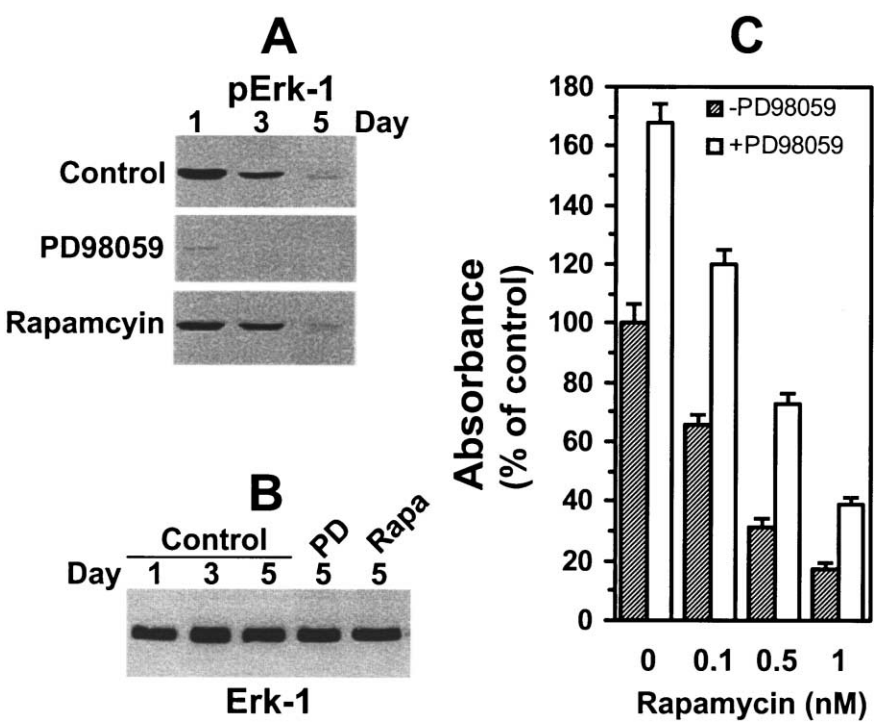

Fig. 5. Rapamycin does not modulate ERK-1 signaling during micromass culture of mesenchymal cells. (A and B) Micromass culture was carried out for the indicated time periods in the presence of vehicle alone as a control, 10 μM PD98059, or 1 nM rapamycin. Activation of ERK-1 was determined using antibody specific to activated ERK-1 and -2 (A), and expression of ERK-1 was examined with anti-ERK-1 antibody by Western blot analysis (B). (C) Chondrogenesis was quantified using cells cultured for 4 days in the presence of the indicated concentrations of rapamycin with or without 10 μM PD98059. The data in (A) and (B) represent the results of a typical experiment of five independent experiments, and (C) represents means ± S.E.M. ($n = 5$). Each point in (C) is $P < 0.01$, compared to the PD98059-untreated.

suggest that the blockage of protein kinase C α activation by rapamycin is one of the mechanisms responsible for the inhibition of chondrogenesis.

3.5. Rapamycin does not modulate ERK-1 phosphorylation during chondrogenesis

Our earlier observation that chondrogenesis required inhibition of ERK-1 activity (Chang et al., 1998; Oh et al., 2000) led us to examine whether rapamycin modulated ERK-1 signaling. Consistent with our previous observation, phosphorylation of ERK-1 decreased as chondrogenesis proceeded (Fig. 5A) without any modulation of its expression (Fig. 5B). The pattern of ERK-1 phosphorylation was not affected by rapamycin (Fig. 5A). However, addition of PD98059 (2-[amino-3-methoxyphenyl]-4H-1-benzopyran-4-one), a specific inhibitor of the ERK-1/-2 upstream kinase (Alessi et al., 1995), blocked ERK-1 phosphorylation (Fig. 5A) and enhanced chondrogenesis both in the absence and presence of rapamycin (Fig. 5C). Therefore, the results suggest that ERK-1 is not involved in the mechanism of rapamycin-induced inhibition of chondrogenesis in differentiating mesenchymal cells.

3.6. Rapamycin inhibits p38 MAP kinase activity during chondrogenesis

Since activation of p38 MAP kinase is required for type II collagen expression and chondrogenesis during the progression from precartilage condensation to cartilage nodule formation (Oh et al., 2000), we investigated the role of rapamycin in the regulation of p38 MAP kinase. In agreement with our previous observation, p38 MAP kinase activity increased as chondrogenesis proceeded (Fig. 6A). Inhibition of p38 MAP kinase with SB203580 (4-[4-fluorophenyl]-2-[4-pyridyl]-1H-imidazole) (Fig. 6B), a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), blocked chondrogenesis as determined by Alcian blue staining (Fig. 6C) or type II collagen staining (data not shown). p38 MAP kinase activity was dramatically inhibited when cells were exposed to rapamycin (Fig. 6B), with concomitant inhibition of chondrogenesis (Fig. 6C). Therefore, the inhibition of p38 MAP kinase by rapamycin appears to parallel the rapamycin-induced inhibition of chondrogenesis.

3.7. Rapamycin inhibits p70 S6 kinase during chondrogenesis

p70 S6 kinase is a ubiquitous serine/threonine kinase that participates in diverse cellular functions and is one of the best-known signaling molecules regulated by rapamycin. For this reason, we investigated p70 S6 kinase activity during chondrogenesis and its regulation by rapamycin. During the chondrogenic differentiation of mesenchymal cells induced by micromass culture, p70 S6 kinase was active throughout the culture period, as evidenced by its phosphorylation status determined by mobility-shift assay. Phosphorylation of p70 S6 kinase was

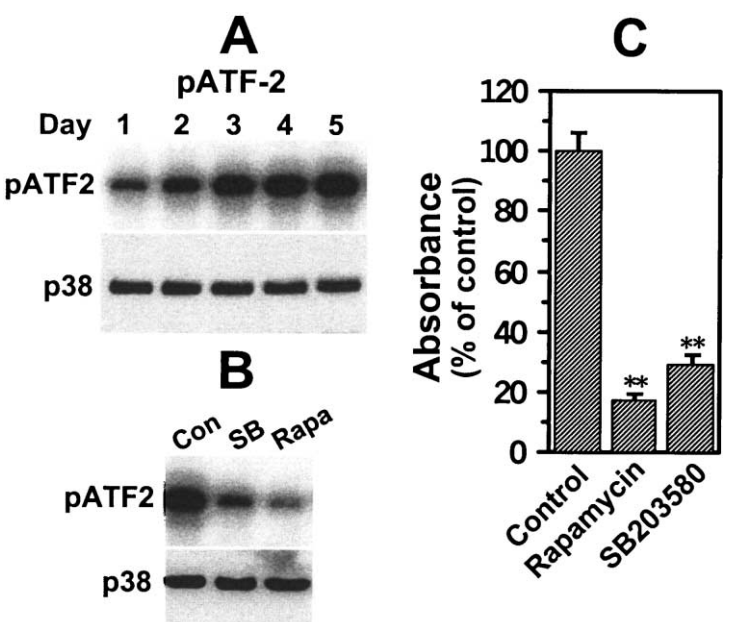

Fig. 6. Rapamycin inhibits p38 MAP kinase activation during chondrogenesis. (A) Cells were cultured for the indicated periods and p38 MAP kinase activity was determined by immune complex kinase assay using ATF-2 as substrate (upper panel). Expression of p38 MAP kinase was examined by Western blot analysis of the same cell lysates (lower panel). (B) Cells were cultured for 5 days in the absence as the control or presence of 10 μM SB203580 or 1 nM rapamycin. p38 MAP kinase activity was determined by immune complex kinase assay (upper panel). Expression of p38 MAP kinase was examined by Western blot analysis of the same cell lysates (lower panel). (C) Chondrogenesis was quantified by Alcian blue staining of cells cultured for 4 days in the absence or presence of 10 μM SB203580 or 1 nM rapamycin. The data in (A) and (B) represent the results of a typical experiment of six independent experiments, and (C) represents means ± S.E.M. ($n = 6$). $^{**}P < 0.01$, compared to the control.

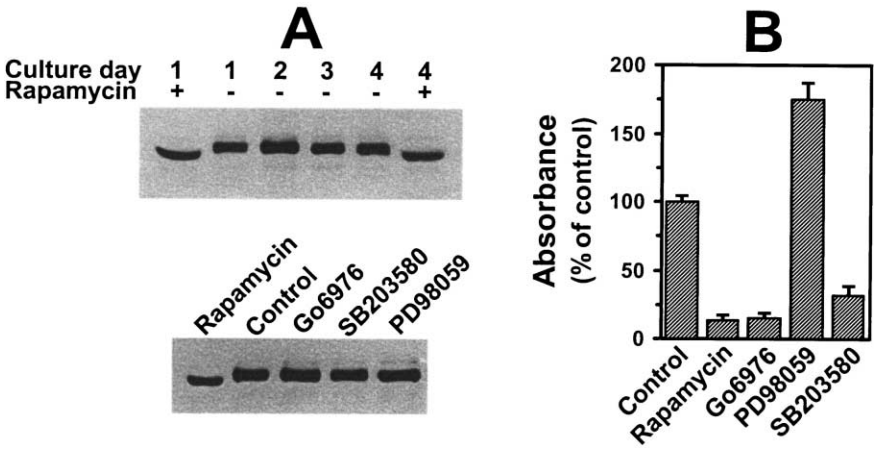

Fig. 7. Inhibition of p70 S6 kinase in rapamycin treated cells. (A) Mesenchymal cells were maintained as micromass cultures for the indicated time periods in the absence or presence of 1 nM rapamycin (upper panel). Mesenchymal cells were cultured for 3 days in the absence (control) or presence of 1 nM rapamycin, 1 μM Go6976, 10 μM PD98059, or 10 μM SB203580. Activation of p70 S6 kinase was determined from the electrophoretic mobility shift in a gel and by Western blot analysis (lower panel). (B) Mesenchymal cells were cultured for 4 days in the absence (control) or presence of 1 nM rapamycin, 1 μM Go6976, 10 μM PD98059, or 10 μM SB203580. Chondrogenesis was quantified by staining sulfated glycosaminoglycan with Alcian blue and measuring the absorbance of bound Alcian blue extract at 600 nm. The data in (A) represent the results of a typical experiment conducted five times, and (B) shows means ± S.E.M. ($n = 5$). Each point in (B) is $P < 0.01$, compared to the control.

completely abolished when mesenchymal cells were treated with 1 nM rapamycin (Fig. 7A, upper panel). However, the phosphorylation state of p70 S6 kinase was not affected by the inhibition of protein kinase C with Go6976, ERK-1 with PD98059, or p38 MAP kinase with SB203580 (Fig. 7A, lower panel) although chondrogenesis was affected under these conditions (Fig. 7B). Therefore, signaling molecules known to regulate chondrogenesis, such as protein kinase C, ERK-1, and p38 MAP kinase, do not appear to be upstream signaling molecules of p70 S6 kinase during the chondrogenic differentiation of mesenchymal cells.

4. Discussion

We investigated in this study whether the immunosuppressant rapamycin affects the chondrogenesis of mesenchymal cells, and found that rapamycin inhibits chondrogenesis by modulating PKCα and p38 MAP kinase signaling pathways. Chondrogenesis is known to be regulated at the stages of cell proliferation, precartilage condensation, and/or cartilage nodule formation (Ahrens et al., 1977; Roark and Greer, 1994; Solursh et al., 1978; Stott et al., 1999). We, therefore, investigated the regulatory mechanisms of rapamycin by dividing chondrogenesis into various stages: (i) proliferation of chondrogenic competent cells, (ii) precartilage condensation, and (iii) progression of precartilage condensation to cartilage nodules. It is well known that rapamycin suppresses cell growth and blocks cell cycle progression from G1 to S phase in some cell types (Brown et al., 1994; Thomas and Hall, 1997). We also observed that 1 nM rapamycin reduced the rate of cell proliferation; however, the effect of rapamycin on chondrogenesis was much more dramatic than its effect on cell proliferation. Additionally, increasing the cell density from 2 to 4×10^7 cells/ml resulted in significantly higher cell numbers even in the presence of rapamycin, while chondrogenesis was still inhibited to the same degree as observed at 2×10^7 cells/ml. Thus, we conclude that the reduction of cell number by rapamycin is not a cause of the blockade of chondrogenesis.

We found in this study that the blockage of chondrogenesis by rapamycin treatment exerted was due to inhibition of the progression from precartilage condensation to cartilage nodule formation. Although the molecular mechanism for cartilage nodule formation is not completely understood yet, we have previously shown that inhibition or down-regulation of protein kinase C or inhibition of p38 MAP kinase or protein kinase A blocks the formation of cartilage nodule from precartilage condensation without any modulation of precartilage condensation (Chang et al., 1998; Oh et al., 2000; Yoon et al., 2000a). Therefore, inhibition of the progression of precartilage condensation to cartilage nodule formation in rapamycin-treated cells is in good agreement with the observation that rapamycin inhibits protein kinase C α and p38 MAP kinase signaling pathways.

The chondrogenesis of mesenchymal cells is regulated by complex protein kinase signaling cascades. Protein kinase C, one of the key signaling molecules active in chondrogenesis, positively regulates the chondrogenic differentiation of mesenchymal cells. It has been demonstrated that the expression and activation of protein kinase C α are essential for the induction of chondrogenesis (Choi et al., 1995; Chang et al., 1998; Yoon et al., 2000b).

The increased expression and activation of PKCα correlates with the decreased activity of ERK-1 during chondrogenesis, while inhibition or down-regulation of protein kinase C α enhances ERK-1 activity, resulting in inhibition of chondrogenesis (Chang et al., 1998; Yoon et al., 2000b). In contrast to ERK-1, p38 MAP kinase activity is increased during chondrogenesis in a PKC-independent manner, and inhibition of p38 MAP kinase activity blocks chondrogenesis (Oh et al., 2000). We have also previously demonstrated that the opposing roles of ERK-1 and p38 MAP kinase in regulating chondrogenesis are played out by the opposite regulation of the expression of cell adhesion molecules at the post-precartilage condensation stage (Oh et al., 2000). In the present study, we examined the effects of the immunosuppressant rapamycin on the chondrogenesis of mesenchymal cells and investigated the rapamycin-induced modulation of cell signaling in an attempt to define the mechanism underlying the rapamycin-induced modulation of chondrogenesis. We were able to show that rapamycin inhibits protein kinase C α and p38 MAP kinase during chondrogenesis, kinases that regulate progression of precartilage condensation to cartilage nodule formation.

Rapamycin blocked the activation of protein kinase C α in differentiating mesenchymal cells without modulating its expression. Since selective down-regulation of protein kinase C α with by thymeleatoxin or inhibition by Go6976 is sufficient to inhibit chondrogenesis (Yoon et al., 2000b), we conclude that the rapamycin-induced inhibition of protein kinase C α signaling was one of the causes for the inhibition of chondrogenesis. Several possible mechanisms exist for the inhibition of protein kinase C α activation by rapamycin. For instance, it has recently been reported that mTOR directly phosphorylates protein kinase C, which is necessary for its activation, and that inhibition of mTOR with rapamycin blocks the phosphorylation and activation of protein kinase C (Parekh et al., 1999; Zeigler et al., 1999). The activation of protein kinase C is a complicated process that requires phosphorylation at three conserved sites, and these phosphorylations act cooperatively to maintain the kinase in an active conformation while still requiring binding to diacylglycerol and phospholipid for activity (Dutil et al., 1998; Le Good et al., 1998: Parekh et al., 2000). Therefore, we deem it possible that inhibition of mTOR by rapamycin blocks the phosphorylation of protein kinase C α that is required for its activation.

Rapamycin does not appear to regulate ERK-1 signaling, since rapamycin treatment did not affect ERK-1 phosphorylation. This observation is consistent with other reports which have shown that rapamycin does not affect ERK signaling (Chung et al., 1994). However, since inhibition or down-regulation of protein kinase C α results in enhanced ERK-1 activation and inhibition of chondrogenesis (Chang et al., 1998; Oh et al., 2000), we expected that inhibition of protein kinase C α signaling by rapamycin treatment would lead to the activation of ERK-1 in order to inhibit type II collagen expression and chondrogenesis. Unexpectedly, we found that the inhibition of protein kinase C α by rapamycin did not alter the phosphorylation status of ERK-1 and that inhibition of ERK-1 by PD98059 enhanced chondrogenesis even in the presence of rapamycin. The results, therefore, suggest that rapamycin inhibits chondrogenesis either independently of the inhibition of protein kinase C α or through an unknown mechanism (other than ERK-1) which is also regulated by protein kinase C α. The mechanism underlying this unexpected observation remains to be elucidated.

Unlike ERK-1 signaling, however, p38 MAP kinase activity was dramatically blocked by rapamycin treatment without any discernible effect on p38 MAP kinase expression. p38 MAP kinase activity increases during differentiation, and inhibition of its activity by SB203580 blocks chondrogenesis (Oh et al., 2000). Therefore, we conclude that inhibition of p38 MAP kinase signaling by rapamycin is just one mechanism leading to inhibition of the expression of type II collagen and chondrogenesis. There are no identified signaling molecules situated between mTOR and p38 MAP kinase. However, the rapamycin-mediated regulation of protein kinase C α signaling appears to be independent of the regulation of p38 MAP kinase signaling, since inhibition or down-regulation of PKCα does not affect p38 MAP kinase activity (Oh et al., 2000). We are currently in the process of investigating signaling pathways leading to the regulation of p38 MAP kinase by rapamycin in chondrifying mesenchymal cells.

The rapamycin-induced regulation of cellular function is mediated by the formation of a complex with the cellular target FK506-binding protein, and the complex binds to a family of kinases, mTOR (Brown and Schreiber, 1996; Chou and Blenis, 1995). One of the best known targeting molecules of mTOR is p70 S6 kinase, which is a ubiquitous serine/threonine kinase participating in diverse cellular functions. Rapamycin is a potent and specific inhibitor of p70 S6 kinase, preventing its activation by all known agonists, by blocking the activation of an upstream kinase mTOR (Brown et al., 1995; Price et al., 1992; Tsai et al., 1993). In this study, we also observed inhibition of p70 S6 kinase in rapamycin-treated mesenchymal cells. However, the phosphorylation of p70 S6 kinase was not affected by the modulation of protein kinase C α, ERK, or p38 MAP kinase signaling, whereas rapamycin treatment inhibited protein kinase C α and p38 MAP kinase. Therefore, it is likely that protein kinase C α and p38 MAP kinase are not upstream signaling molecules of p70 S6 kinase. However, it remains to be determined whether p70 S6 kinase is an upstream signaling molecule of PKCα and p38 MAP kinase and if rapamycin-induced inhibition of p70 S6 kinase is responsible for the inhibition of protein kinase C α and p38 MAP kinase.

In summary, rapamycin negatively regulates the chondrogenic differentiation of chick limb bud mesenchymal cells. Rapamycin exerted its regulatory effects during the

progression from precartilage condensation to cartilage nodule formation by modulating protein kinase C α and p38 MAP kinase signaling pathways.

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