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Retinoic Acid Inhibits Chondrogenesis of Mesenchymal Cells by Sustaining Expression of N-Cadherin and its Associated Proteins

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Abstract Retinoic acid (RA) is a well-known regulator of chondrocyte phenotype. RA inhibits chondrogenic differentiation of mesenchymal cells and also causes loss of differentiated chondrocyte phenotype. The present study investigated the mechanisms underlying RA regulation of chondrogenesis. RA treatment in chondrifying mesenchymal cells did not affect precartilage condensation, but blocked progression from precartilage condensation to cartilage nodule formation. This inhibitory effect of RA was independent of protein kinase C and extracellular signal-regulated protein kinase, which are positive and negative regulators of cartilage nodule formation, respectively. The progression from precartilage condensation to cartilage nodule requires downregulation of N-cadherin expression. However, RA treatment caused sustained expression of N-cadherin and its associated proteins including α- and β-catenin suggesting that modulation of expression of these molecules is associated with RA-induced inhibition of chondrogenesis. This hypothesis was supported by the observation that disruption of the actin cytoskeleton by cytochalasin D (CD) blocks RA-induced sustained expression of cell adhesion molecules and overcomes RA-induced inhibition of chondrogenesis. Taken together, our results suggest RA inhibits chondrogenesis by stabilizing cell-to-cell interactions at the post-precartilage condensation stage. J. Cell. Biochem. 89: 837–847, 2003. © 2003 Wiley-Liss, Inc.

Key words: mesenchymal cells; chondrogenesis; micromass culture; retinoic acid; N-cadherin

Cartilage formation in developing limb buds of embryos is initiated by chondrogenic differentiation of mesenchymal cells [Sandell and Adler, 1999; DeLise et al., 2000]. Micromass culture of embryonic mesenchymal cells is a useful model system to study chondrogenesis. Dissociated limb bud mesenchymal cells undergo differentiation to chondrocytes in vitro when cells are cultured at high density [Ahrens et al., 1977]. Both in vivo and in vitro chondrogenesis is initiated by precartilage condensation via cell-to-cell adhesion [DeLise et al., 2000]. N-cadherin, a key Ca^{2+}-dependent cell adhesion molecule, mediates cell-to-cell adhesion in association with α- and β-catenin [Nagafuchi, 2001; Conacci-Sorrell et al., 2002]. Indeed, perturbation of N-cadherin-mediated cell adhesion with neutralizing antibody or expression of a dominant negative N-cadherin inhibits precartilage condensation [Oberlender and Tuan, 1994; DeLise and Tuan, 2002a]. Chondrogenic differentiation is completed by progression from precartilage condensation to cartilage nodules. In contrast to the requirement for N-cadherin and its associated molecules during precartilage condensation, downregulation of these adhesion molecules accompanies cartilage nodule formation [Chang et al., 1998; DeLise et al., 2000; Oh et al., 2000]. It has been shown that sustained expression of N-cadherin contributes to inhibition of chondrogenesis [Tufan and Tuan, 2001; DeLise and Tuan, 2002b; Tufan et al., 2002a,b], indicating that strict temporal regulation of N-cadherin expression

Abbreviations used: CD, cytochalasin D; ERK, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; RA, retinoic acid.

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is required for chondrogenesis of mesenchymal cells.

Regulation of chondrogenesis, including expression of N-cadherin, is regulated by complex protein kinase signaling cascades involving protein kinase C (PKC) [Chang et al., 1998], extracellular signal-regulated kinase (ERK) [Chang et al., 1998; Oh et al., 2000], p38 mitogen-activated protein (MAP) kinase [Oh et al., 2000; Yoon et al., 2000a], and protein kinase A (PKA) [Yoon et al., 2000b]. PKCα positively affects chondrogenesis of mesenchymal cells by regulating expression of cell adhesion molecules such as N-cadherin, fibronectin, and its receptor α5β1 integrin, leading to progression from precartilage condensation to cartilage nodules [Chang et al., 1998]. PKC-dependent regulation of chondrogenesis is exerted via MAP kinase subtype ERK-1 [Chang et al., 1998; Oh et al., 2000]. The pattern of ERK-1 activation is inversely related to expression and activity of PKCα. It was found that increased expression and activation of PKC was required for downregulation of ERK-1 activity, which correlated with induction of chondrogenic differentiation of mesenchymal cells. In addition, inhibition or downregulation of PKC (conditions that inhibit chondrogenesis) resulted in activation of ERK-1, while inhibition of ERK-1 with PD98059 blocked the inhibitory effects of PKC downregulation on chondrogenesis [Chang et al., 1998; Oh et al., 2000]. In contrast to ERK-1, p38 MAP kinase conversely regulates chondrogenesis at post-precartilage condensation stages by modulating expression of adhesion molecules in a manner independent of PKCα [Oh et al., 2000].

Retinoic acid (RA), a vitamin A derivative, is a well-known regulator of cartilage and skeletal formation [Underhill and Weston, 1998]. RA has an effect on anteroposterior and proximodistal axis formation in developing limb buds [Tamura et al., 1997]. Modulation of RA availability during chondrogenesis has a profound impact on the skeleton [Kochhar, 1973; Kwasigroch and Kochhar, 1980], suggesting that chondrogenesis is particularly sensitive to RA. Indeed, RA has been shown to inhibit chondrogenic differentiation of mesenchymal cells and to cause loss of differentiated chondrocyte phenotype [Biddulph et al., 1988; Jiang et al., 1995; Tsonis et al., 1996; Cash et al., 1997; Weston et al., 2000,2002]. However, molecular mechanisms underlying RA regulation of chondrogenesis are not clearly understood.

In the present study, we investigated the mechanism of RA inhibition of mesenchymal cell chondrogenesis. Because the PKCα promoter contains an RA response element [Desai et al., 1999], and RA has been shown to inhibit PKCα activity [Radominska-Pandya et al., 2000], we first focused on the role of PKCα and its downstream signaling molecule ERK-1. We further investigated the role of N-cadherin and its associated proteins in inhibition of chondrogenesis, since both PKCα and ERK exert their effects through regulation of N-cadherin expression. We report here that RA inhibits chondrogenesis by blocking progression from precartilage condensation to cartilage nodule formation, and that this is achieved through sustained expression of N-cadherin and its associated proteins in a manner independent of PKCα and ERK signaling.

MATERIALS AND METHODS

Micromass Culture of Mesenchymal Cells

Mesenchymal cells were isolated from the whole wing buds of Hamburger–Hamilton stage 23/24 chicken embryo limbs from fertilized White Leghorn eggs. The cells were suspended at a density of 2.0×10^7 cells/ml in Ham's F-12 medium and spotted into culture dishes in 15 μl drops. 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 bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin, either in the absence or presence of various all-*trans*-RA and other pharmacological reagents, as described in the text. Chondrogenesis was determined by examining the expression of type II collagen and accumulation of sulfated glycosaminoglycans by Alcian blue staining [Chang et al., 1998; Oh et al., 2000].

PKC Assay

PKC activity was determined by in vitro kinase assays described previously [Kim et al., 2002]. Total cell lysates were prepared from cells at day 5 micromass culture by extracting proteins with a lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.01% SDS, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) containing inhibitors of proteases (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml pepstatin A, 20 μg/ml aprotinin, and 20 μg/ml

leupeptin) and phosphatases (50 mM NaF and 1 mM Na_3VO_4). PKCα was immunoprecipitated from total cell lysates using anti-PKCα monoclonal antibodies (Transduction Laboratories, Lexington, KY), and the immune complexes were collected with protein A-Sepharose beads. After washing with lysis buffer, beads were resuspended in 30 μl kinase reaction buffer (40 mM HEPES, pH 7.4, 1 mM EGTA, 20 mM $MgCl_2$, 50 μM ATP, 2 mM dithiothreitol, [γ-^{32}P] ATP, and protease and phosphatase inhibitors) containing 1 μg myelin basic protein as a PKCα substrate. Following incubation for 30 min at 30 °C, the reaction was stopped by addition of 4 × Laemmli's sample buffer and boiled. The samples were resolved by electrophoresis, and phosphorylation of substrate was determined by autoradiography. Where indicated, in some experiments PKCα was ectopically overexpressed using adenovirus containing PKCα cDNA. Cultures were either infected with wild-type adenovirus or adenovirus with PKCα cDNA in serum-free Ham's F-12 medium for 3 h at a range from 0.1 to 1.5 pfu/cell. Following infection, medium was replaced with Ham's F-12 medium containing 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin, either in the absence or presence of 25 nM RA.

ERK Assay

Proteins from mesenchymal cells were extracted with a buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS) containing inhibitors of proteases and phosphatases. ERK-1 activation was then determined by immunoblot analysis using an antibody specific for phosphorylated ERK-1 (New England Biolabs, Beverly, MA), as previously described [Chang et al., 1998; Oh et al., 2000].

Immunoblot Analysis

Whole cell lysates were prepared by extracting proteins using a buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS) supplemented with protease and phosphatase inhibitors. Proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 3% nonfat dry milk in Tris-buffered saline. Type II collagen and Sox-9 was detected using antibodies purchased from Chemicon (Temecula, CA) and Santa Cruz (Santa Cruz, CA), respectively. PKCα was detected using an anti-PKCα antibody (BD Transduction Laboratories). Expression of adhesion molecules was determined using antibodies purchased from the following sources: mouse anti-mouse N-cadherin monoclonal antibody from BD Transduction Laboratories, rabbit anti-human α-catenin polyclonal antibody from Santa Cruz, and mouse β-catenin monoclonal antibody from BD Transduction Laboratories. We have previously shown that the antibodies against type II collagen, ERK-1, pERK, PKCα, N-cadherin, and β-catenin specifically react with avian proteins [Chang et al., 1998; Oh et al., 2000, Yoon et al., 2000a,b; Ryu et al., 2002]. Other antibody such as anti-Sox-9 also specifically recognizes avian protein based on the size of detected protein. Blots were developed using a peroxidase-conjugated secondary antibody and an ECL system.

Immunocytochemistry

Cells from whole wing bud cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 40 min at room temperature. The cells were washed and incubated for 1 h with an antibody against type II collagen, N-cadherin, α-catenin, and β-catenin or 100 mg/ml of biotinylated PNA (Vector Laboratories Inc., Burlingame, CA). Binding was visualized by developing with VECTASTAIN ABC and DAB substrate solution kits from Vector Laboratories Inc., according to the procedure recommended by the manufacturer.

RESULTS

Ra Inhibits Chondrogenesis of Mesenchymal Cells at the Post-Precartilage Condensation Stage

Micromass culture of mesenchymal cells induced chondrogenesis, as determined by type II collagen expression (Fig. 1A). As expected, RA treatment in chondrifying mesenchymal cells dramatically blocked expression of type II collagen and accumulation of sulfated proteoglycans in a dose-dependent manner (Fig. 1A,B). Consistent with the inhibition of type II collagen expression, levels of SOX9, a potent activator of the chondrocyte-specific enhancer of the pro alpha 1 (II) collagen gene [Lefebvre et al., 1997; DeLise et al., 2000], was decreased by the treatment of cells with RA (Fig. 1A). RA treatment did not affect precartilage condensation as determined by peanut agglutinin staining [Aulthouse and Solursh, 1987], but blocked

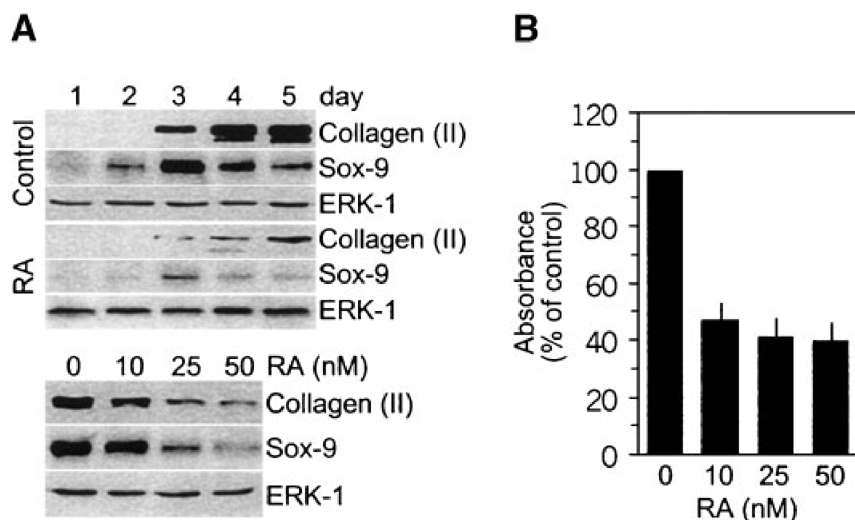

Fig. 1. RA inhibits chondrogenesis of mesenchymal cells. **A**: Mesenchymal cells were micromass cultured for the indicated time periods in the absence or presence of 25 nM RA (**upper panel**). Alternatively, cells were cultured in the presence of the indicated concentrations of RA for 5 days (**lower panel**). Expression of type II collagen and SOX-9 was determined by immunoblot analysis. ERK-1 was detected as a loading control of proteins. **B**: Mesenchymal cells were cultured for 4 days in the presence of the indicated concentrations of RA and accumulation of sulfated proteoglycans was quantified by Alcian blue staining. The data in A represent results of a typical experiment from four independent experiments with similar results, and in B represent mean values and SD (n = 6).

cartilage nodule formation as determined by Alcian blue staining (Fig. 2A), indicating that RA inhibits progression from precartilage condensation to cartilage nodule. Because precartilage condensation, which depends on cell packing, requires proliferation of chondrogenic competent cells, and RA is known to modulate proliferation of certain cell types [Rosewicz et al., 1996; Cho et al., 1997], we examined whether the inhibitory action of RA on chondrogenesis is due to inhibition of proliferation of chondrogenic competent cells. Although RA treatment reduced chondrogenesis up to 47% of the control level, it did not affect proliferation

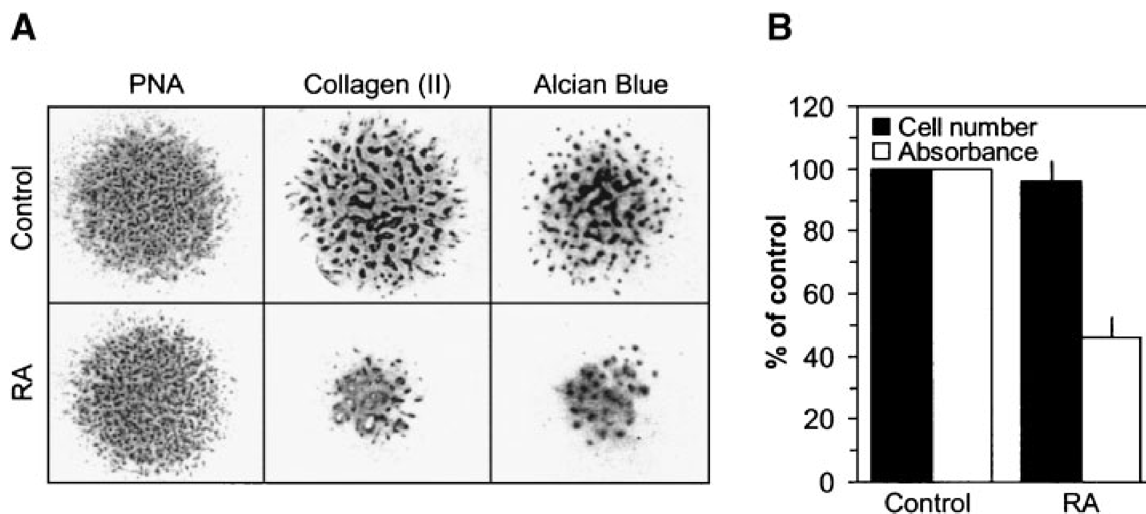

Fig. 2. RA inhibits progression from precartilage condensation to cartilage nodule formation. **A**: Mesenchymal cells were cultured in the absence or presence of 25 nM RA for 2 days and stained with PNA (**left-panel**). The cells were cultured for 4 days and stained for type II collagen (**middle panel**) or with Alcian blue (**right panel**). The data represent a typical result from four independent experiments with similar results. **B**: Mesenchymal cells were cultured in the absence or presence of 25 nM RA for 4 days. The number of cells was counted (trypan blue exclusion) and chondrogenesis was quantified by Alcian blue staining. The data in B represent mean values and SD (n = 6).

of chondrogenic competent mesenchymal cells (Fig. 2B). The above results indicate that RA inhibits progression from precartilage condensation to cartilage nodule formation, independent of any effect on cell proliferation.

PKCα and ERK Signaling do not Mediate RA-Induced Inhibition of Chondrogenesis

RA is known to modulate PKC activity [Radominska-Pandya et al., 2000], and our previous study indicated that PKCα acts as a positive regulator of chondrogenesis [Chang et al., 1998]. This led us to examine whether inhibition of chondrogenesis by RA treatment is mediated by PKCα signaling. Expression of PKCα was dramatically increased during chondrogenesis, consistent with our previous observations [Chang et al., 1998]. RA dose-dependently decreased PKCα expression (Fig. 3A), suggesting decreased PKCα expression contributes to RA-induced inhibition of chondrogenesis. To examine this possibility, mesenchymal cells were infected with an adenovirus that causes ectopic expression of PKCα (Fig. 3B). Unexpectedly, overexpression of PKCα (Fig. 3B, lower panel) did not affect RA-induced inhibition of type II collagen expression (Fig. 3B, lower panel) and accumulation of sulfated proteoglycans (Fig. 3B,C). Ectopically expressed PKCα was enzymatically active both in the absence and presence of RA (Fig. 3D), indicating that PKCα signaling does not mediate RA-induced inhibition of chondrogenesis.

It has been shown that PKC regulates chondrogenesis by inhibiting ERK signaling, which is downregulated during chondrogenesis [Chang et al., 1998; Oh et al., 2000]. We examined whether ERK signaling is involved in RA-induced inhibition of chondrogenesis. Consistent with previously reported results [Chang et al., 1998], phosphorylation of ERK-1 was reduced during chondrogenesis (Fig. 4A, upper panel). However, RA treatment had no effect on ERK activation (Fig. 4A, lower panel). In addition, inhibition of ERK with PD98059 did not affect RA-induced inhibition of type II collagen expression (Fig. 4A, lower panel) and accumulation of sulfated proteoglycans (Fig. 4B), although PD98059 treatment in control cultures significantly enhanced accumulation of sulfated proteoglycan (Fig. 4C). Taken together, the above results indicate that the inhibitory effect of RA on chondrogenesis is not mediated by either PKCα or ERK signaling pathways.

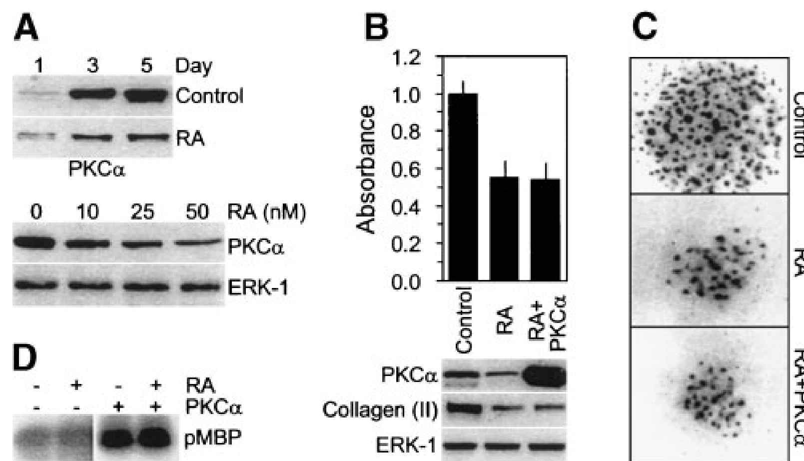

Fig. 3. PKCα does not mediate RA-induced inhibition of chondrogenesis. **A**: Mesenchymal cells were maintained as micromass cultures in the absence or presence of 25 nM RA for the indicated times (**upper panel**), or for 5 days in the presence of the indicated concentrations of RA (**lower panel**). Expression of PKCα was determined by immunoblot analysis. ERK-1 was detected as a loading control of proteins. **B** and **C**: Mesenchymal cells were infected with either adenovirus carrying the control vector (Control and RA) or adenovirus carrying the PKCα cDNA (RA + PKCα). After 3 h of infection, cells were cultured in the absence (Control) or presence of 25 nM RA (RA and RA + PKCα) for 4 days. Accumulation of sulfated glycosaminoglycans was determined by Alcian blue staining (C) and quantified by measuring absorbance at 600 nm (B, **upper panel**). Expression of PKCα and type II collagen was determined by immunoblot analysis (B, **lower panel**). ERK-1 was detected as a loading control of proteins. **D**: Mesenchymal cells were infected with either adenovirus carrying the control vector (−) or adenovirus carrying the PKCα cDNA (+). After 3 h of infection, cells were cultured in the absence (−) or presence of 25 nM RA (+). PKCα activity was determined by immune complex kinase assay. The data represent the results of a typical experiment or mean values and SD (n = 4).

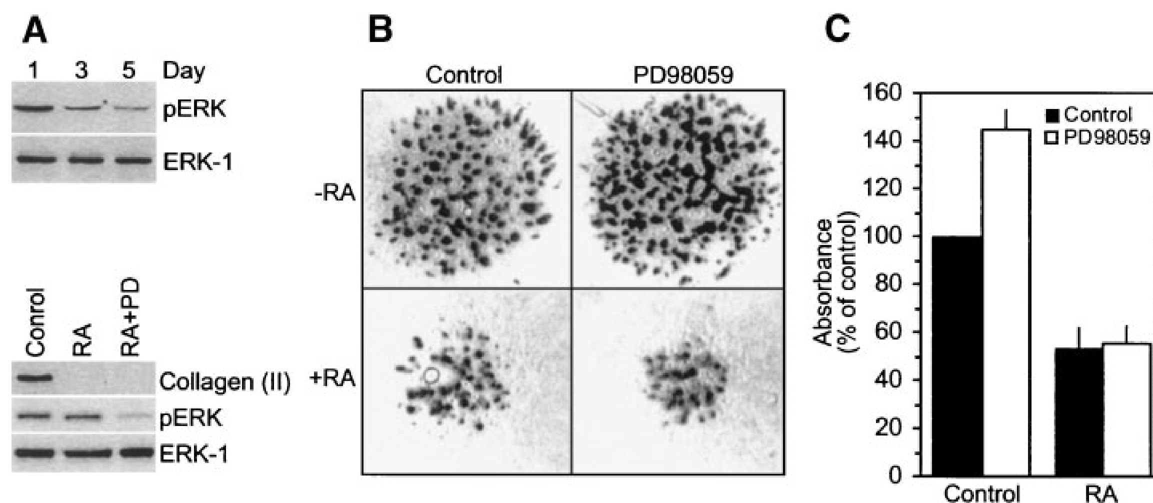

Fig. 4. ERK does not mediate RA-induced inhibition of chondrogenesis. Mesenchymal cells were maintained as micromass cultures for the indicated times in the absence or presence of 25 nM RA and/or 10 μM PD98059. Expression of type II collagen, ERK-1, and phosphorylation of ERK-1 was determined by immunoblot analysis (**A**). Cartilage nodule formation was determined by Alcian blue staining (**B**). Chondrogenesis was quantified by measuring absorbance of the Alcian blue extract at 600 nm (**C**). The data represent results of a typical experiment or mean values and SD from five independent experiments.

RA-Induced Inhibition of Inhibits Chondrogenesis is Associated With Modulation of Expression of N-Cadherin and its Associated Proteins

Both precartilage condensation and progression to cartilage nodule formation depend on N-cadherin, which regulates cell-to-cell adhesion. Therefore, we examined the effect of RA on expression and distribution of N-cadherin and its associated proteins, including α- and β-catenin. Immunocytochemical study in control cultures showed that type II collagen staining was positive only in cartilage nodules that were composed of differentiated chondrocytes (Fig. 5). In contrast to the pattern of type II collagen, staining of N-cadherin, α-catenin, and β-catenin was negative in cartilage nodules but positive on the edge of cartilage nodules and inter-nodular areas. RA treatment significantly reduced the number of nodules that are strongly stained for N-cadherin, α-catenin, and β-catenin (Fig. 5). Immunoblot analysis indicated that expression of N-cadherin, α-catenin, and β-catenin was high in 1-day-old cultures, and their expression decreased as chondrogenesis proceeded (Fig. 6A). However, RA treatment caused sustained expression of these molecules up to day 5 (Fig. 6A), and the effect was dose-dependent (Fig. 6B). Taken together, these results suggest that sustained expression of adhesion molecules is associated with the RA-induced inhibition of chondrogenesis.

To further elucidate the role of N-cadherin expression in RA-induced inhibition of chondrogenesis, cells were treated with cytochalasin D (CD), an actin cytoskeleton-disrupting agent that causes cells to form round shapes and enhances chondrogenesis [Zanetti and Solursh, 1984; Lim et al., 2000]. CD treatment in control cultures enhanced cartilage nodule formation (Fig. 7A) and type II collagen expression (Fig. 7B), which was accompanied by decreased levels of N-cadherin and α- and β-catenin. CD treatment also blocked RA-induced sustained expression of N-cadherin and α- and β-catenin, and overcame the inhibition of cartilage nodule formation and type II collagen expression (Fig. 7). The above data further suggest that RA-induced modulation of cell adhesion molecule expression is associated with inhibition of chondrogenesis.

DISCUSSION

Although RA is known to inhibit chondrogenic differentiation of mesenchymal cells both in vivo and in vitro, the mechanisms underlying this property are not fully understood. The present study examined RA-induced inhibition of chondrogenesis induced by micromass culture of embryonic mesenchymal cells. We demonstrated that RA blocks chondrogenesis by inhibiting progression of precartilage condensation to cartilage nodule formation and the inhibition of chondrogenesis is correlated with

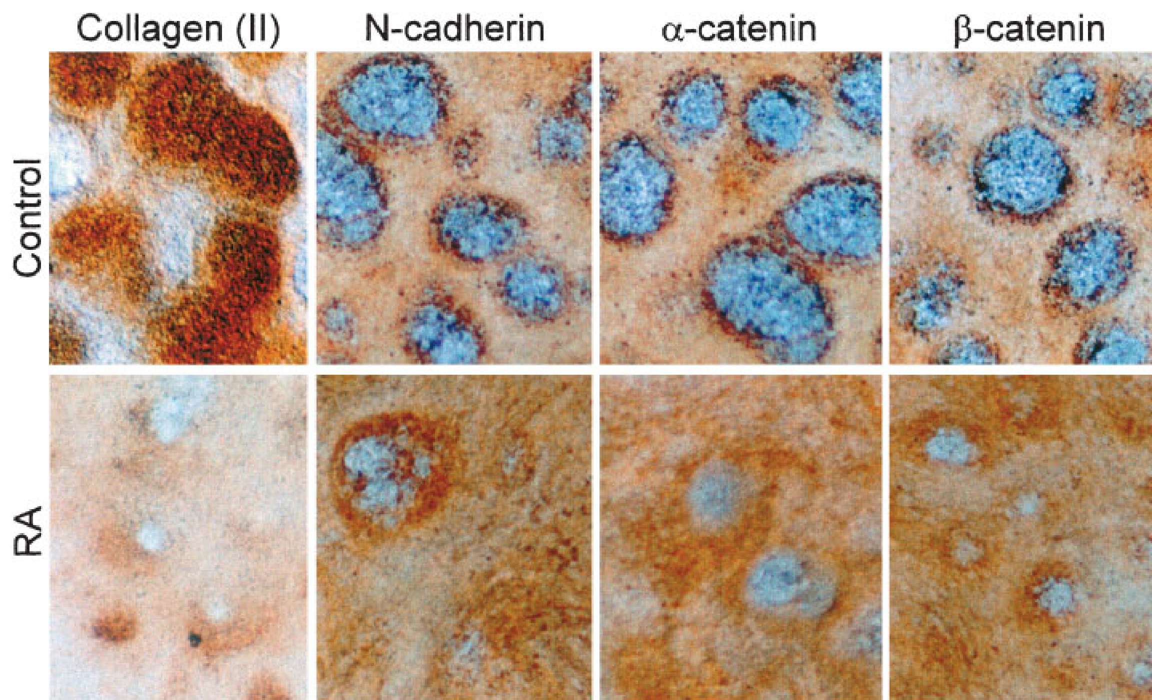

Fig. 5. Distribution pattern of type II collagen, N-cadherin, and α- and β-catenin in chondrifying mesenchymal cells. Mesenchymal cells were cultured for 4 days in the absence or presence of 25 nM RA. The cells were fixed with paraformaldehyde and stained for type II collagen, N-cadherin, and α- and β-catenin, and visualized by developing with VECTASTAIN ABC and DAB substrate solution kits. The data represent a typical result carried out more than five times with similar results.

the sustained expression of N-cadherin and its associated proteins α- and β-catenin in a manner independent of PKCα and ERK signaling.

Precartilage condensation via N-cadherin-mediated cell-to-cell adhesions is a prerequisite step for initiation of chondrogenesis of mesenchymal cells. For instance, inhibition of N-cadherin function by neutralizing antibody or expression of a dominant negative form blocks precartilage condensation to inhibit chondrogenesis [Oberlender and Tuan, 1994; DeLise and Tuan, 2002a]. However, progression from precartilage condensation to cartilage nodules requires downregulation of N-cadherin [DeLise

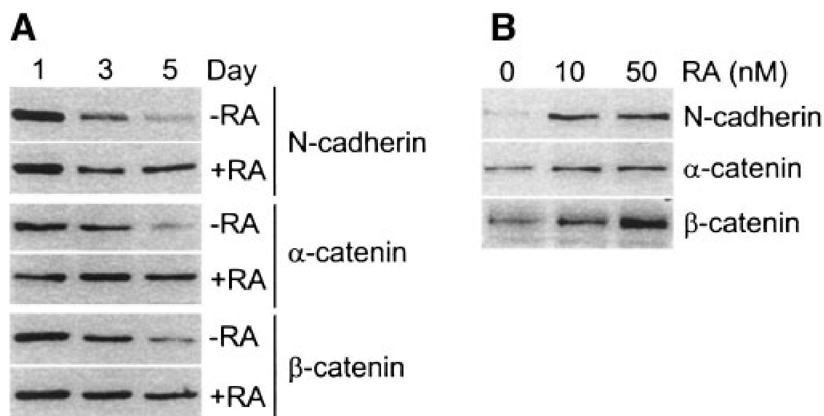

Fig. 6. RA causes sustained expression of N-cadherin and α- and β-catenin. **A** and **B**: Mesenchymal cells were maintained as micromass cultures for the indicated times in the absence or presence of 25 nM RA (A), or for 5 days in the presence of the indicated concentrations of RA (B). Expression of N-cadherin and α- and β-catenin was determined by immunoblot analysis. The data represent results of a typical experiment conducted more than five times with similar results.

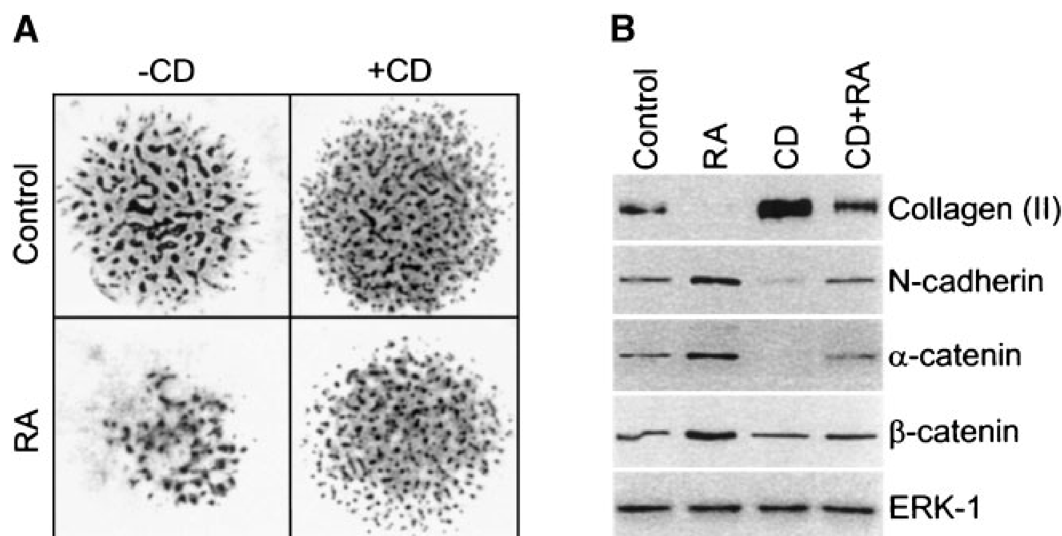

Fig. 7. Cytochalasin D (CD) inhibits RA-induced sustained expression of N-cadherin and α- and β-catenin, and overrides inhibition of chondrogenesis. Mesenchymal cells were cultured for 4 days in the absence or presence of 25 nM RA and/or 50 nM CD. The cells were stained with Alcian blue (**A**). Expression of type II collagen, N-cadherin, and α- and β-catenin was determined by immunoblot analysis (**B**). ERK-1 was detected as a loading control of proteins. The data represent a typical result of experiment carried out more than five times with similar results.

et al., 2000]. Indeed, conditions that block downregulation of N-cadherin, such as exposure of cells to Wnt signals, inhibit chondrogenesis [Tufan and Tuan, 2001; Tufan et al., 2002a,b]. Furthermore, it has been shown that overexpression of N-cadherin inhibits cartilage nodule formation without any effects on precartilage condensation [DeLise and Tuan, 2002a], suggesting that sustained expression of N-cadherin and inhibition of chondrogenesis is not independent and parallel events caused by RA. Therefore, it seems likely that RA-induced sustained expression of N-cadherin and its associated proteins contributes to the inhibition of chondrogenesis by stabilizing cell-cell adhesion. However, it remains to be elucidated whether sustained expression of N-cadherin is the major mechanism by which RA exerts its negative effects on chondrogenesis. Nevertheless, the ability of RA to cause sustained expression of N-cadherin in mesenchymal cells is consistent with the observation that RA treatment causes increased expression of N-cadherin, increased cell-to-cell adhesion, and the recruitment of cytoplasmic β-catenin to the membrane in epithelial and breast cancer cells [Vermeulen et al., 1995].

Cadherin-dependent cell adhesion can be regulated in several ways, including changes in the composition of the adhesion complex, phosphorylation of components in the complex, and alterations in the interaction of the complex with the actin cytoskeleton [Nagafuchi, 2001; Conacci-Sorrell et al., 2002]. Thus, to evaluate the role of sustained expression of N-cadherin and its associated proteins in inhibition of chondrogenesis, cells were treated with the actin depolymerizing agent, CD, which changes actin organization and functions as a signal in the modulation of chondrocyte phenotype [Zanetti and Solursh, 1984; Lim et al., 2000]. As expected, CD treatment enhanced chondrogenesis, and CD co-treatment rescued RA-induced inhibition of chondrogenesis with a concomitant downregulation of N-cadherin and α- and β-catenin. Based on the ability of CD that positively regulates chondrogenesis, it is currently unclear whether the apparent reversal of RA-induced inhibition of chondrogenesis by CD treatment is due to its positive regulation of differentiation or its ability to suppress N-cadherin expression. However, our data are consistent with the observations that N-cadherin expression plays an inhibitory role in the progression from precartilage condensation to cartilage nodules.

We have previously shown that cartilage nodule formation from precartilage condensation requires complex protein kinase signaling cascades involving PKC, ERK, p38 MAP kinase,

and PKA [Chang et al., 1998; Oh et al., 2000; Yoon et al., 2000a,b]. Chondrogenesis requires increased expression and activity of PKCα that regulates progression of precartilage condensation to cartilage nodules [Chang et al., 1998]. A possible involvement of PKCα in RA regulation of chondrogenesis has been suggested by the observation that the PKCα promoter contains an RA response element [Desai et al., 1999], and that RA binds PKCα directly to decrease PKCα activity [Radominska-Pandya et al., 2000]. However, our data indicated that sustained expression of N-cadherin and its associated proteins is not due to modulation of PKCα signaling, and also indicate that PKCα signaling does not mediate RA-induced inhibition of chondrogenesis. Although RA treatment in chondrifying mesenchymal cells caused partial inhibition of PKCα expression, ectopic expression of PKCα did not override RA-induced inhibition of chondrogenesis. In addition, the activity of ectopically expressed PKCα was not affected by RA treatment, although it may be that inhibition of PKCα activity requires 1 μM or higher concentrations of RA [Radominska-Pandya et al., 2000]. We also demonstrated that RA-induced inhibition of chondrogenesis is independent of ERK signaling. This is consistent with the observation that PKCα exerts its regulatory effects by inhibiting ERK-1 signaling [Chang et al., 1998].

In addition to PKC and ERK signaling, chondrogenesis requires PKA and p38 MAP kinase activity. We have previously shown that ERK and p38 MAP kinase regulate chondrogenesis by conversely modulating expression of N-cadherin at the post-precartilage condensation stage in a manner independent of PKCα [Oh et al., 2000]. Our previous results also indicated that PKA activation is an upstream event required for PKCα activity at the post-precartilage condensation stage [Yoon et al., 2000b]. Although we did not examine the role of PKA and p38 MAP kinase in RA regulation of chondrogenesis, it has been demonstrated that these two signaling molecules are involved in RA-mediated inhibition of chondrogenesis. For example, inhibition of RAR that is necessary and sufficient for chondrogenesis results in activation of PKA and p38 MAP kinase, and chondrogenesis induced by inhibition of RAR is blocked by inhibition of PKA and p38 MAP kinase. In contrast, inhibition of chondrogenesis induced by RAR activation is rescued by activation of PKA and p38 MAP kinase [Weston et al., 2002]. This suggests that activation of PKA and p38 MAP kinase, rather than PKCα and ERK signaling, mediates RA regulation of chondrogenesis. However, it is not clear at present whether RA-induced sustained expression of N-cadherin and its associated proteins is regulated by PKA and p38 MAP kinase signaling. PKA and p38 MAP kinase signaling has been shown as a requirement for RAR-mediated gene repression, suggesting that sustained expression of N-cadherin is not regulated by PKA and p38 kinase. In addition, our previous observation that PKA exerts its regulatory effect by stimulating PKCα [Yoon et al., 2000b], and our current observation that PKCα does not mediate RA-induced inhibition of chondrogenesis, further suggest that sustained expression of N-cadherin is not mediated by PKA and p38 MAP kinase. Therefore, it appears that RA-induced inhibition of chondrogenesis is due to sustained expression of N-cadherin and its associated proteins that occur independently of signaling by PKA, PKC, ERK, and p38 MAP kinases.

Micromass culture of mesenchymal cells to induce chondrogenesis uses mesenchymal cell population derived from the entire chick embryo limb bud. Consequently, the mesenchymal cell population is heterogeneous containing not only chondrogenic progenitor cells but also myogenic cells and other mesenchymal and fibroblastic cells. Therefore, it may be possible that any effects on chondrogenesis observed in this study such as inhibition of chondrogenesis by RA treatment, rescue of RA-induced inhibition of chondrogenesis by CD treatment, and unresponsiveness of chondrogenesis by PKCα expression could be due to changes in the behavior of other cell population rather than chondrogenic progenitor cells. However, based on the observation that RA treatment does not affect early event of chondrogenesis such as precartilage condensation and that there is no detectable changes in myosin heavy chain expression, a marker for myogenesis (data not shown), the effects on chondrogenesis appear to be due to modulation of the behavior of chondrogenic cells.

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