Inhibition of Chondrogenesis by Cytochalasin D in High Density Micromass Culture of Chick Mesenchymal Cells: Its Effects on Expression of α-Smooth Muscle Actin and P-cadherin

Jeong Ah Yoo, Su-Jung Park, Shin-Sung Kang, and Tae Kyu Park*

Department of Biology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

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Mesenchymal cells from the leg buds of stage 24-chick embryos differentiated into chondrocytes when plated at high density. Treatment of high density micromass culture of chick mesenchymal cells with cytochalasin D (CD, 2 μM for 24 h) resulted in inhibition of chondrogenesis. CD treatment was found to affect the expression of the contractile protein α-smooth muscle actin (α-SM actin). In control cultures, α-SM actin uniformly expressed from culture day 2, but the CD-treated cells induced expression of α-SM actin from the first day of culture followed by a continuous increase. Expression of pan-cadherin (P-cadherin) decreased as chondrogenesis proceeded in the control culture, whereas the CD-treated cells showed sustained expression. These results propose a close connection of chondrogenic differentiation with expression of α-SM actin and P-cadherin.

Mesenchymal cells in high density micromass culture differentiate into cartilage nodules of the chick limb buds (Ahren et al., 1977; Tacchetti et al., 1992). Chondrogenesis, the differentiation of mesenchymes into chondrocytes, is cell density dependent and is characterized mainly by the syntheses of type II collagen and large cartilage proteoglycan which have been used to define their specific differentiated phenotype (Muller et al., 1977; Kimura et al., 1981). It has been suggested that limb mesenchymal cells at low density undergo chondrogenesis when they are exposed to the actin disrupting drug, cytochalasin D (CD) (Zanetti and Solursh, 1984). Also, dedifferentiated chondrocytes are able to redifferentiate by CD treatment (Newman and Watt, 1988; Loty et al., 1995). Therefore, it is well known that CD induces chondrogenesis. However, we consider that it is not appropriate to apply the ability of CD to the overall chondrogenesis because chondrogenesis is cell density dependent. Furthermore, although it has been clearly established that disruption of actin cables in CD-treated low density culture of mesenchymal cells controls morphology of cultured limb bud cells and induces chondrogenesis (Zanetti and Solursh, 1984), it is uncertain whether the disruption of actin cables also results in the induction of chondrongenesis in CD-treated high density cultures. In addition, it is not clear what kind of actin isoforms are involved.

In the present study, we investigated the effect of CD on the chondrogenesis of high density culture of chick mesenchymal cells. CD treatment of high density cultures resulted in inhibition of chondrogenic differentiation in contrast to its promotion effect on low density cultures, and led to increased expression of α-SM actin and sustained expression of P-cadherin.

Materials and Methods

Micromass culture of mesenchymal cells

Mesenchymal cells were derived from the distal tips of Hamburger-Hamilton stage 24 embryo leg buds (Hamburger and Hamilton, 1951) of fertilized white Leghorn chicken eggs and were micromass cultured as described previously (Park et al., 1990). Briefly, leg bud tips were dissected and mesenchymal cells were enzymatically dissociated in Ca^{2+}-, Mg^{2+}-free saline G containing 0.1% trypsin (Sigma, St. Louis, MO) and 0.1% collagenase (Sigma). The cells were suspended at a density of 2 x 10^7 cells/ml in Ham's F-12 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL, Life Technologies, NY). The cells were plated in five drops of 15 μl to 35 mm Corning culture dishes and incubated for 1 h at 37℃ under 5% CO_2 to allow attachment. The cells were maintained in culture medium either in the absence or presence of 2 μM of CD. The

* To whom correspondence should be addressed.
Tel: 82-53-950-5345; Fax: 82-53-953-3066
E-mail: tkpark@knu.ac.kr

medium was changed daily.

Quantitative analysis of chondrogenesis

Chondrogenesis was quantified as previously described (Park et al., 1990). Briefly, the cultures were rinsed once with Hank's Balanced Salt Solution (HBSS) and fixed in Kahle's Fixative for 10 min. To visualize the sulfated cartilage matrix, cells were stained with Alcian blue at pH 1.0 overnight. Alcian blue bound to sulfated glycosaminoglycans was extracted with 4 M guanidine-HCl, and quantified by reading the absorbance of Alcian blue extract at 600 nm.

Western blot analysis

Total cell lysates were prepared from micromass cultured cells for various time periods by extracting proteins with a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate). Proteins were separated by 7.5% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 3% nonfat dry milk in Tris-buffered saline, and probed with respective antibodies. Expression of proteins was determined using monoclonal antibodies against α- and β-skeletal actin, α- smooth muscle actin, P-cadherin, catenin-α, -β and -γ (Sigma). The membranes were washed three times with Tris-buffered saline and incubated with secondary antibody-conjugated with horseradish peroxidase (Sigma), and visualized using enhanced chemiluminescence detection kit (Amersham, UK).

Immunofluorescence assay

Cultured Cells were rinsed with several changes of phosphate-buffered saline (PBS) and fixed in ice-cold methanol for 5 min, then rehydrated in PBS at room temperature for 10 min. Cells were then incubated for overnight at 4℃ with antibody against α-SM actin diluted 1/10 in PBS. After rinsing with PBS four times the cells were incubated with TRITC-conjugated goat anti-mouse antibody for 20 min. After rinsing with PBS four times, they were mounted in vectashild. Immunofluorescence was analyzed with a fluorescence microscopy (Zeiss Jenaval, Germany).

Results and Discussion

Mesenchymal cells micromass cultured at high density spontaneously underwent differentiation to chondrocytes, whereas chondrogenic differentiation of the CD (2 μM for 24 h)-treated cultures was severely inhibited (Fig. 1A & B). It was reported that in low density micromass culture (5.0×10^6 cells/ml) of limb bud mesenchymal cells, disruption of actin cytoskeleton by CD, that causes cells to become round shaped, induced chondrogenesis (Zanetti and Solursh, 1984). On the contrary,

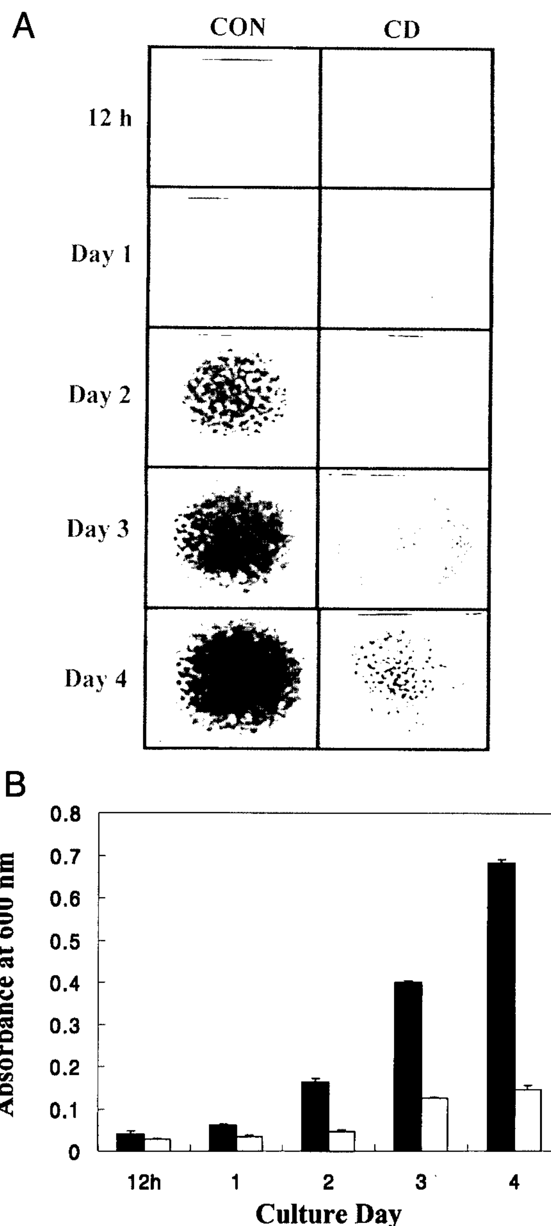

Fig. 1. Effects of CD treatment on the chondrogenesis of high density micromass culture. Mesenchymal cells were cultured in the absence (■) or presence of 2 μM CD for 24 h (□). A, The cells were fixed with Kahl's Fixative and stained with Alcian blue (pH 1.0). B, Chondrogenesis was quantified by staining of sulfated glycosaminoglycans with Alcian blue (pH 1.0) and reading the absorbance of bound Alcian blue extract. Each bar indicates the mean value ± s.d. from three independent cultures.

in our high density culture, the CD treatment led to inhibition of chondrogenesis.

To identify the factors leading to the inhibition of chondrogenesis in the CD-treated high density cultures, we analyzed protein expression levels of the actin isoforms by Western blotting (Fig. 2). The protein expression levels of α- and β-skeletal actin did not change in either control or CD-treated cultures through-

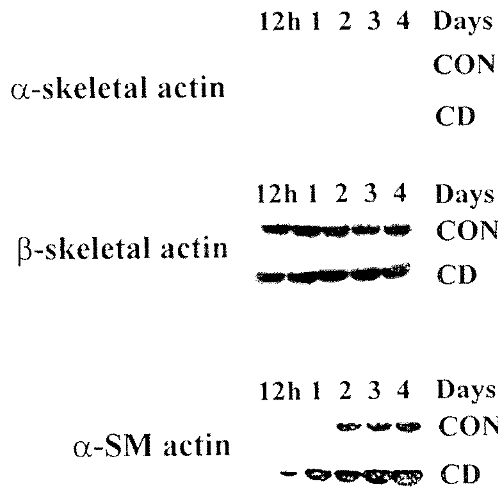

Fig. 2. Effects of CD on the expression of actin isoforms during chondrogenesis of high density micromass culture. Mesenchymal cells were micromass cultured in the absence (CON) or presence of 2 μM CD for 24 h (CD) and cultured in a plain medium for indicated periods and harvested. Cell lysates were subjected to SDS-PAGE, and analyzed by Western blotting using antibody against each indicated protein.

out culture periods. On the other hand, the expression of α-SM actin showed a different pattern. α-SM actin was expressed from the culture day 2 which was sustained throughout the culture periods in the control, while larger amounts of α-SM actin appeared from the first day of culture which continuously increased in the CD-treated cultures. These results suggest that increased expression of α-SM actin by CD is related to inhibition of chondrogenesis. We next examined whether the expression of α-SM actin was also increased in the CD-treated low density cultures. When determined by immunofluorsence microscopy using a monoclonal antibody directed against α-SM actin, the morphological change induced by the CD treatment (2 μM for 24 h) at low density cultures clearly showed α-SM actin staining at the peripheral region of cells, and each cell maintained a round configuration (Fig. 3). Consequently, this result indicates that the expression of α-SM actin is promoted by CD treatment regardless of cell density. In this study, however, we could not compare the expression levels of α-SM actin induced by CD between low and high density cultures. Thus, we conceived that inhibition of chondrogenesis by increased expression of α-SM actin may be due to the presence of another function(s) of α-SM actin.

The α-SM actin, a contractile actin isoforms was expressed prevalently in vascular smooth muscle cells but transiently in certain non-muscle cells, particularly myofibroblasts (Petridou and Marsur, 1996; Marsur et al., 1999). It was reported that CD promotes chondrogenesis by disruption of actin cables leading to modification of cytoskeleton architecture and subsequent remodeling of the cells (Zanetti and Solursh, 1984; Newman and Watt, 1988; Loty et al., 1995). Several

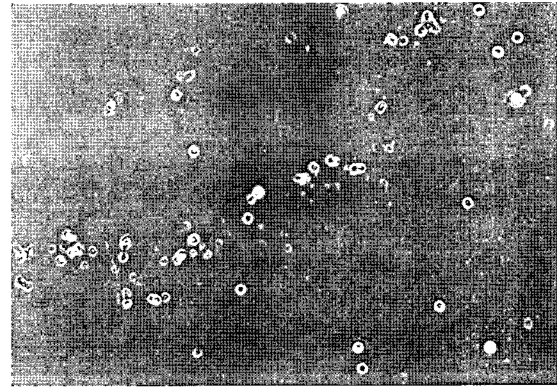

Fig. 3. Immunofluorescence microscopy of CD-treated low density cultures stained with anti-α-SM actin antibody. Cells were cultured at a density of 5 x 10^6 cells/ml in the presence of CD (2 μM for 24 h). Cells were fixed with ice-cold methanol and immunostained with anti-α-SM actin antibody. Cells were photographed using fluorescence microscope at x 400. Note that a-SM actin expressed at the peripheral region of cells and each cells maintained a round configuration.

other reports have presented that the major reorganizable cytoskeletal components by external stimuli during chondrogenesis are microfilaments, polymers of β-actin subunits, rather than microtubules and intermediate filaments (Benya et al, 1988; Brown and Benya, 1988; Benjamin et al., 1994). However, as shown in Fig. 2, CD treatment inhibited chondrogenesis in our high density cultures. These results suggest that the effect of reorganization of cytoskeleton architecture on chondrogenesis highly depends on the cell culture conditions such as cell density.

It was also reported that the presence of P-cadherin at the cell-cell borders of myofibroblasts might provide a site of insertion of actin filaments (Petridou and Marsur, 1996). We, therefore, explored the expression of P-cadherin in both control and CD-treated cultures. The CD-treated cultures showed a sustained expression level of P-cadherin throughout culture periods, while the control culture showed an initial peak level followed by a decrease (Fig. 4). Taken together, our results suggest that sustained expression of P-cadherin along with increased expression of α-SM actin may be associated with inhibition of chondrogenesis in high density cultures of mesenchymal cells. To confirm the

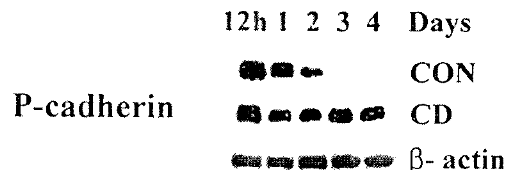

Fig. 4. Effects of CD on the expression of P-cadherin during chondrogenic differentiation. Culture conditions were the same as in Fig. 2. Mesenchymal cells were micromass cultured in the absence (CON) or presence of 2 μM CD for 24 h and cultured in a plain medium for indicated periods. Cell lysates were subjected to SDS-PAGE, and analyzed by Western blotting using an antibody against anti-pan-cadherin. β-actin was used as the mark for loading control.

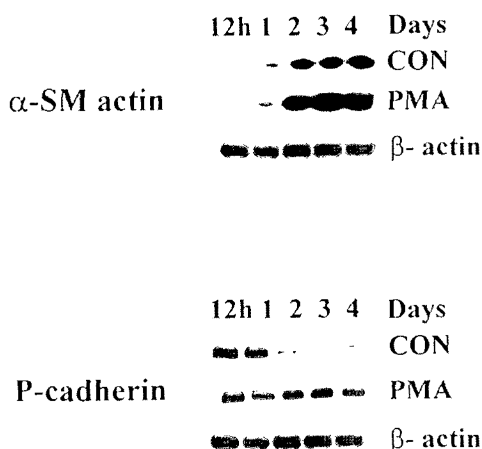

Fig. 5. Effects of PMA on the expression of α-SM actin and P-cadherin during chondrogenic differentiation of high density micromass culture. Mesenchymal cells were micromass cultured in the absence (CON) or presence of 100 nM PMA (PMA) for the indicated time period and harvested. Expression of proteins was determined by Western blot analysis using antibody against anti-α-SM actin or anti-pan-cadherin. β-actin was used as the mark for loading control.

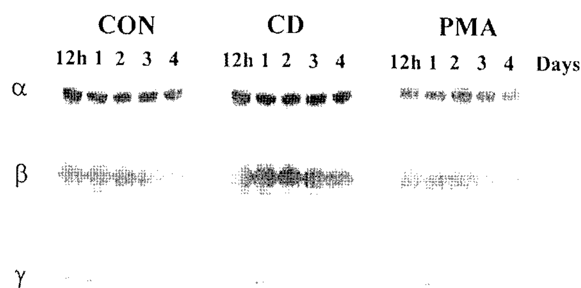

Fig. 6. Expression pattern of catenin isoforms during chondrogenesis. of high density micromass culture. Mesenchymal cells were micromass cultured in the absence (CON) or presence of 2 μM CD for 24 h and then in a plain medium (CD) or presence of 100 nM PMA (PMA) for the indicated time periods and harvested. Expression of proteins was determined by Western blot analysis using antibody against anti-catenin for α, β and γ, respectively.

above results, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a known chondrogenic inhibitor, on the expression of α-SM actin and P-cadherin. When the cells were treated with 100 nM PMA, the expression levels of α-SM actin increased and P-cadherin displayed a similar expression pattern as that of the CD-treated cultures (Fig. 5). PMA also showed no changes in the expression of the other cytoskeletal proteins, α- and β-skeletal actin (data not shown). Therefore, it can be inferred that increased expression of α-SM actin accompanied by sustained expression of P-cadherin is related to inhibition of chondrogenic differentiation. Because cadherin molecules associated with actin-based cytoskeletal elements (Hirano et al., 1987; Pavalko and Otey, 1994; Danjo and Gipson, 1998; Quinlan and Hyatt, 1999), and the reduction of P-cadherin was observed during chondrogenesis, and expression of α-SM actin did not increase (Fig. 2 & 4), these results indicate a negative-relationship of chondrogenesis with the association of P-cadherin to actin-based cytoskeleton.

Catenins, the cadherin-associated proteins, are known to regulate cadherin function and several studies have reported a correlation between cadherin and catenins (Johnson et al., 1993; Knudsen et al., 1995; Hazan et al., 1997). To further assess the role of P-cadherin in the regulation of chondrogenesis, we next investigated changes in expression of catenins in CD-treated or PMA-treated high density cultures. There were no significant changes in the amounts of catenin -α, -β and -γ throughout the culture periods either in control, or CD-treated, or PMA-treated cultures (Fig. 6). Thus, this implies that modulation of P-cadherin may not be associated with the catenin molecules.

In this study, we demonstrated increased expression of α-SM actin coupled with sustained expression of P-cadherin when chondrogenic differentiation of high density micromass culture was inhibited by CD. Although it is not yet clear whether the inhibiting role of CD in chondrogenesis is due to a change in cell morphology by modulation of expression of α-SM actin and P-cadherin, it is an intriguing possibility that the association of α-SM actin and P-cadherin may have modulatory effect on chondrogenesis.

Acknowledgements

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