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A STUDY ON CHONDROGENIC POTENTIAL IN MANDIBULAR AND LIMB BUD MESENCHYMAL CELLS OF HUMAN EMBRYOS : A POSSIBLE ROLE OF PROTEIN KINASE C

Yoon-Ah Kook, Eun-Cheol Kim, Sang-Cheol Kim

We have examined the *in vitro* stage-related chondrogenic potential of human mandibular and limb bud mesenchyme cells using micromass culture. Our results indicate that limb bud mesenchyme cells as early as stage 16 by Carnegie system (37 days), well before the initiation of *in vivo* chondrogenesis, have chondrogenic potential which is expressed in micromass culture. These results are correlated with stage-related chondrogenic potential of human limb bud *in vivo* as a result of Alcian blue staining. The proliferation of chondrogenic cells increased in the first 3 days after culture and then decreased. These results were correlated with the cell cycle analysis of which the number of G_0/G_1 phase increased markedly after 3 days of culture, while the percentage of cells in S phase was decreased. On the other hand, it was rarely differentiated in the mandible.

We examined the effects of two PKC modulators such as phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC, and staurosporine (STSN), an inhibitor of PKC. PMA inhibited the chondrogenesis, whereas STSN promoted the chondrogenesis in a dose dependent manner. In addition, PMA exerted no inhibitory effect when the cells were pretreated for 24 h with STSN, implying that the chondrogenic events might be settled at an early step *in vitro* and PKC may act as a negative modulator. Collectively, these results demonstrate, for the first time, the stage-related chondrogenic potential of human mandibular and limb bud mesenchyme cells and the role of PKC during chondrogenesis *in vitro & in vivo*.

Key words : Human Embryos, Mandible, Limb bud, Micromass culture, PKC, Chondrogenesis

The underlying malocclusions and dentofacial deformities are often related to variations in the craniofacial development. The development and growth of the tissue in the craniofacial skeleton is a complicated process involving a number of changes in the cellular interactions as well as transient stages with changes in morphological appearance.

The two major pathways of bone development involve intramembranous or endochondral osteogenesis. During endochondral bone formation, mesenchymal condensation initially leads to the formation of a cartilaginous template, which is replaced by bony structures at a later stage[5].

Chondrogenesis is one of the first overt cell differentiation events in the development of the vertebrate embryonic limb[25]. A crucial event in cartilage differentiation *in vivo* and *in vitro* is a transient cellular condensation or aggregation process in which prechondrogenic mesenchymal cells become closely juxtaposed to one another prior to depositing a cartilage matrix[12]. During this process a cell-cell interaction, cell shape change, or some other event occurs which is necessary to trigger the chondrogenic differentiation of the cells. Regulating events occurring during condensation result in the secretion of

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cartilage-specific macromolecules such as type II collagen and sulfated proteoglycans[12,16,25].

Chondrogenesis, like many other developmental processes, is regulated by environmental signals apparently including those transmitted through the extracellular milieu and those received by direct surface contact with other cells[12,25]. It was demonstrated that protein kinase C (PKC), the major cellular receptor for phorbol ester, is thought to play a pivotal role in signal transduction by coordinating receptor-mediated hydrolysis of phosphoinositides with a variety of cellular functions such as protein phosphorylation and induction of gene expression[4,18]. Studies utilizing cultured chick limb bud chondrogenic cells showed that chondrogenesis requires Ca^{2+} and involves PKC as a regulator[3,22,27].

Although the roles of tissue interactions during chondrogenesis of mandibular ectomesenchymal cells and the origin of the cells within the mandibular arch are known[10,20], there is little information on the biochemistry and molecular biology of mandibular chondrogenesis. Since Meckel's cartilage is morphologically similar to other hyaline cartilages, it is assumed that events leading to chondrogenesis and regulation of extracellular matrix proteins are similar to those of other hyaline cartilages[15]. However, these cells have different origins than their counterparts in the developing limb. In addition, a potential involvement of PKC on the formation of the cartilage during chondrogenesis in human mandibular and limb bud was not properly defined. In this study, we are going to determine whether there are any specific differences in the mechanisms of chondrogenesis, pattern formation and gene regulation in the mandibular arch.

We have used cell cultures to examine the cellular heterogeneity and developmental potential of the neural crest-derived mandibular and mesodermally derived limb bud mesenchymal cell population in human embryos. Furthermore, we examined the developmental potential of human mandibular and limb bud mesenchymal cell population and the possible role of PKC during chondrogenesis by using micromass cultures.

MATERIALS AND METHODS

Human Embryos

Thirty-five embryos (dated according to the criteria cited between stage 13, 28th post-conceptual day, and stage 20, 51th post-conceptual day) were examined. The developmental stage of an embryo was determined according to the cranium-rump length.

Reagents

Phorbol 12-myristate 13-acetate (PMA), staurosporine (STSN), collagenase, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). $Na_2{}^{35}SO_4$ and [^{3}H]thymidine were from Amersham. Thirty-five mm diameter Petri dishes were purchased from Nunc Inc. (Naperville, IL). Hams F-12, Hanks's balanced salt solution (HBSS), and fetal bovine serum (FBS) and other culture reagents were purchased from Life technologies (Gaithersburg, MD).

Histologic examination

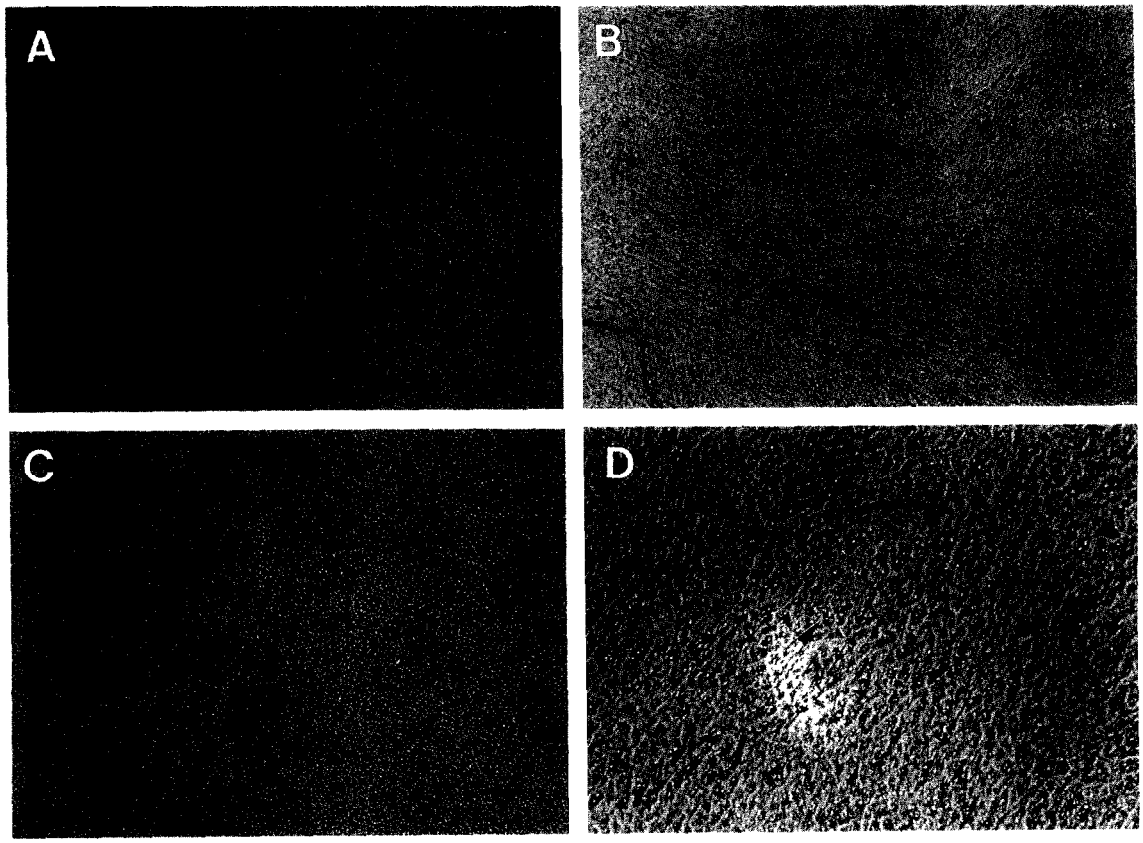
The samples were fixed in neutralized buffered formalin for 1 day at room temperature and embedded in paraffin, 5μm sections were cut, stained with Hematoxylin & Eosin, Alcian Blue, and examined by light microscope.

Micromass Cultures

The micromass culture techniques are essentially those previously developed for the study of limb bud cells from chick embryos[1,2]. Developing human embryonic mandible and limb buds from Carnegie stages 13-20 were isolated in HBSS containing 10% FBS. Mandible and Limb buds were dissociated in HBSS containing 0.2 % trypsin-0.1 % collagenase for 20 min at 37℃ and the resulting cell suspension was filtered through two layers of No. 20 Nitex to remove multicellular aggregates and, after counting, 30 ml of a cell suspension containing 3 x 10^5 single cells were placed in tissue culture dishes. After cell attachment, 2 ml of F12 culture medium containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (antibiotic/ antimycotic), 150-200 μg/ml ascorbic acid, and 10 % FBS were added. The media was replaced every 24h. Cultures were examined and photographed daily.

Staining and Quantitation of Cartilage Formation

For routine quantitation of cartilage nodules, micromass cultures grown for 1-5 days were fixed in



A : Comtrol, B : PMA, C : STSN(x40), D : STSN(x100)

Fig. 1. Micromass cultures of human limb bud mesenchyme cells. Whole mount of 4-day cultures from Carnegie stages 13/14(A), 16/17(B), and 20/21(C) of human limb bud. Cultures were fixed and stained with Alcian blue at pH 1 and then photographed at x 25 using brightfield optics.

Kahles fixative (2 % acetic acid, 12 % formaldehyde, and 30 % ethanol in D/W) for 10 min, rinsed twice with 0.1 N NaCl and stained with 0.5 % Alcian blue (pH 1.0). The number of Alcian blue-staining nodules was determined microscopically using camera lucida [14]. After counting the nodules, the Alcian blue was extracted from stained cells for 6 h with 1 ml of 6 M guanidine-HCl, and then the absorbance at 650 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia)[14]. In some experiments, the rate of [^{35}S]sulfate incorporation into glycosaminoglycans was measured as described[9].

DNA Synthesis

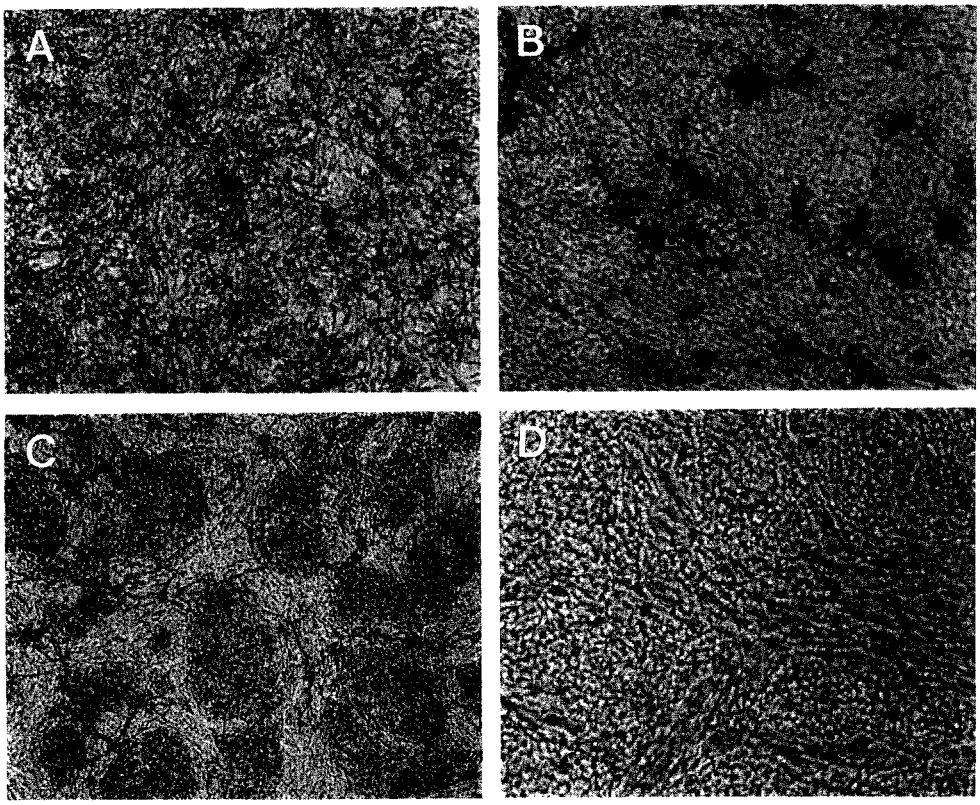
[^{3}H]thymidine (1 μCi) was added to cultured cells for 4 h. The cells were rinsed twice with ice-cold HBSS and then 1 ml of 10 % trichloroacetic acid was added. After 10 min, the precipitates were rinsed twice with 10 % trichloroacetic acid, dissolved in 0.3 N NaOH containing 0.1 % SDS, and counted in a liquid scintilation counter (Tricarb 1500, Packard).

Cell Cycle Analysis

Approximately 10^6 cells per experimental condition were harvested, washed with phosphate-buffered saline, and resuspended in 250 μl of the sample buffer. To this cell suspension, 0.1 % Nonidet P-40 and 50 μg/ml propidium idodide, final concentrations, were sequentially added and the content of DNA per cell was estimated by flow cytometry (Becton-Dickinson, Rutherford, NJ).

RESULTS

Stage-Related Chondrogenesis Of Human Mandibular and Limb Bud Mesenchymal Cells



A : Comtrol, B : PMA, C : STSN (x40), D : STSN (x100)

Fig. 2. Micromass cultures of human mandible mesenchyme cells. Whole mount of 4-day cultures from Carnegie stages 13/14(A), 16/17(B), and 20/21(C) of human mandible. Cultures were fixed and stained with Alcian blue at pH 1 and then photographed at x 25 using brightfield optics.

In Vitro & In Vivo

When dissociated human limb bud mesenchymal cells from early developmental stages (stages 13-14) were placed in micromass cultures, focal aggregates were not formed after seeding (Fig. 1A). However, at stage 16-17, the cells were morphologically homogeneous in the limb bud and focal aggregates were formed by 48 h after seeding. During the second 24 h period of micromass cultures, the cells were subsequently differentiated into cartilage nodules and the nodules expanded in the area to form larger masses of cartilage over the next 3-5 days in culture (Fig. 1B). At stage 20-21 the limb bud mesenchyme was no longer morphologically homogeneous. Distinct bone formation and skeletal primordia could be recognized. In addition, the limb bud cells isolated from stage 20-21 revealed no characteristic chondrogenic potential to posses (Fig. 1C). To assess whether the *in vitro* stage-related chondrogenic potential of human limb bud is correlated with the *in vivo* state, we examined the expression of chondrogenic cells *in vivo* by Alcian blue staining. As can be seen in Figure 1, Alcian blue positive cells were observed after stage 16-17 embryos, but not at stage 13-14 embryos, indicating that the chondrogenic potential may be initiated after stage 16 in human limb bud.

We also compared the chondrogenic potential between mandibular and limb bud mesenchymal cells. As shown in Figures 1 and 3, cells isolated from the limb bud mesenchyme showed significantly more differentiation than the cells isolated from the mandible (stage 16-17). It was rarely differentiated in the mandible.

In human mandibular mesenchymal cells from early developmental stages (stage 13-14), focal aggregates were not formed after seeding (Fig. 2A). On the other hand, the cells from stage 16-17 were morphologically homogeneous in the limb bud and focal aggregates were also formed by 48 h after the seeding. The cells were hot differentiated into cartilage nodules over the next 3-5 days in culture(Fig. 2B). The limb bud mesenchyme was no longer morphologically homogeneous at stage 20-21.

To assess whether the *in vitro* stage related

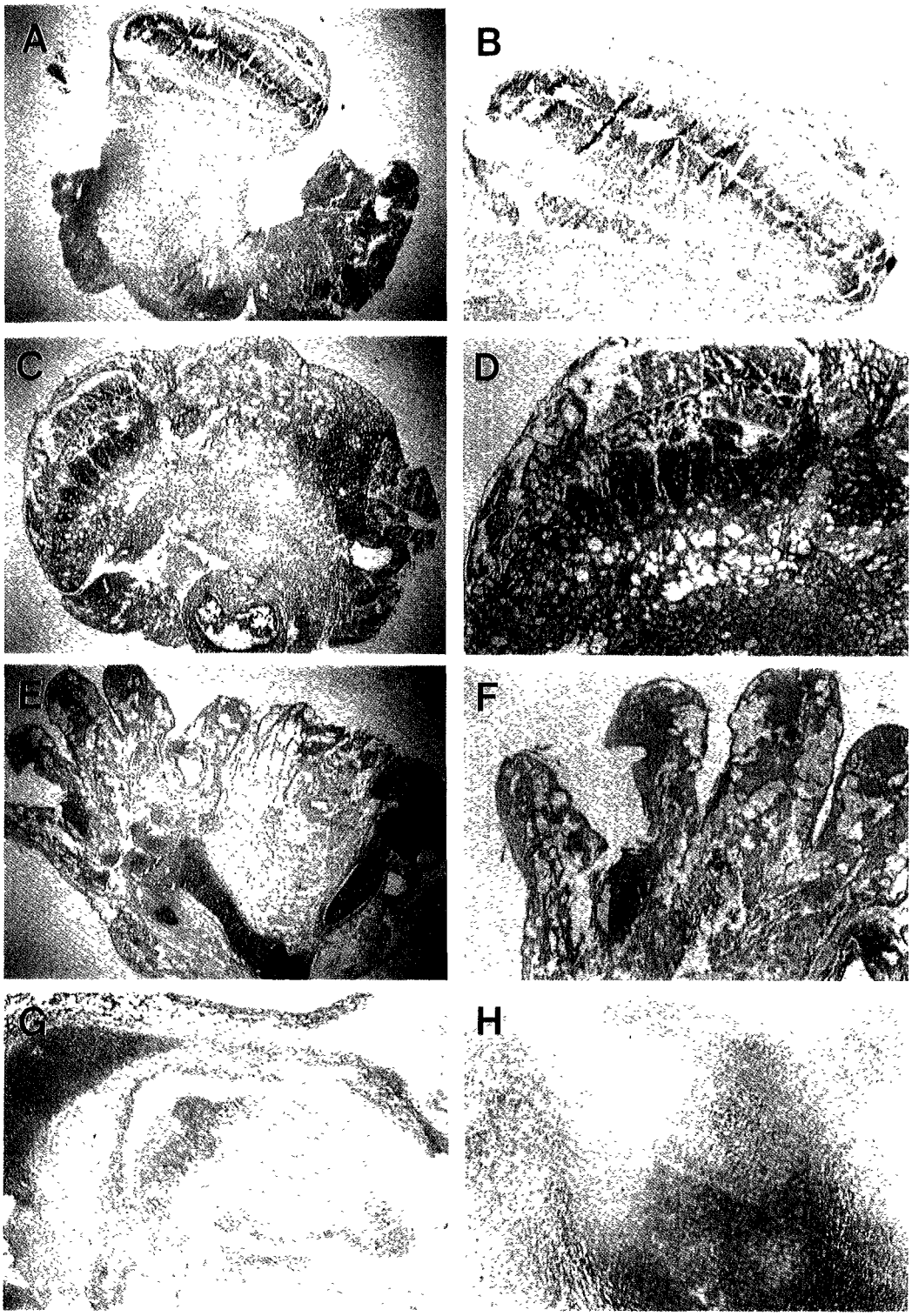


Fig. 3. Lateral section through body of human limb bud Human limb buds from Carnegie stages 13/14 (A and B), 16/17(C and D) and 20/21 (E and F) were fixed and embedded in paraffin. Human mandible from Carnegie stages 13/14 (G), and 16/17(H) were fixed and embedded in paraffin. Tissue sections were stained with Alcian blue and then restained with hematoxylin. Photography was taken at x 40(A, C, E, and G) and x 100 (B, D, F and H).

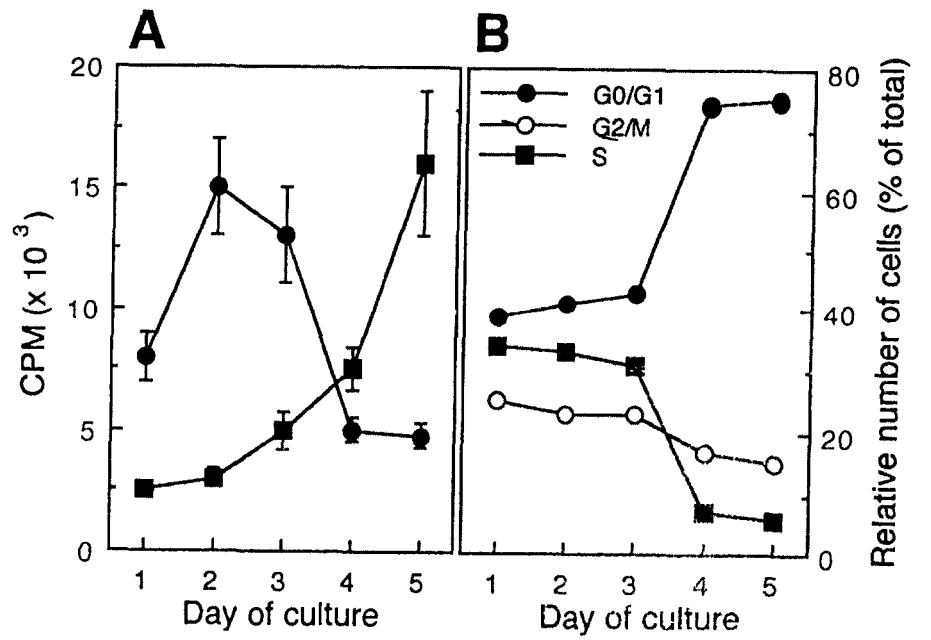


Fig. 4. Proliferation and differentiation of human limb bud mesenchyme cells during cultures *in vitro* (A) The rate of [^{35}S] sulfate and [^{3}H]thymidine incorporation into cells Values are the means SD of three experiments (B) Cell cycle analysis during chondrogenesis in human limb bud.

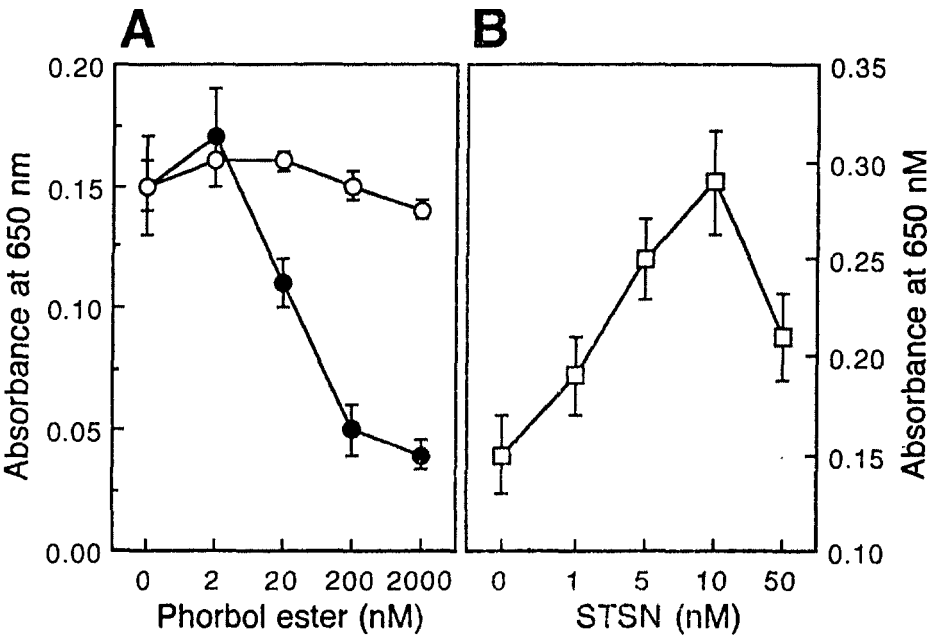


Fig. 5. Dose-dependent effects of PKC modulators on the chondrogenesis *in vitro* Each culture was supplemented with various concentrations of derivatives of phorbol ester or STSN throughout the culture period and assayed at day 4 as described in Materials and Methods (A) Dose-dependent effects of α-PDD and PMA on human limb bud chondrogenesis. (B) Dose-dependent effect of STSN on chondrogenesis. Values are the means SD of three experiments.

chondrogenic poteintial of human mandible and limb bud is correlated with the *in vivo* state, we examined the expresssion of chondrogenic cells *in vitro* by Alcian blue staining. As can be seen in Fig. 3, Alcian blue positive cells were observed after stage 16-17 embryos, but not at stage 13-14 embryos, indicating that the chondrogenic potential may be initiated after stage 16in human mandible and limb bud.

Proliferation and Differentiation of Chondrogenic Cells

As shown in Fig. 4A, the rapid [^{3}H]thymidine incorporation into the cells occurred between day 1 and day 3 in culture and then decreased after day 4. However, the overall patterns of the amount of alcian blue bound to sulfated glycosaminoglycans (Fig. 1B) and the rate of [^{35}S]sulfate incorporation into cell layer macromolecules were similar to those in morphological differentiation which become apparent after day 3. These results were correlated with the cell cycle analysis (Fig. 4B). In the first 3 days after culture, the per

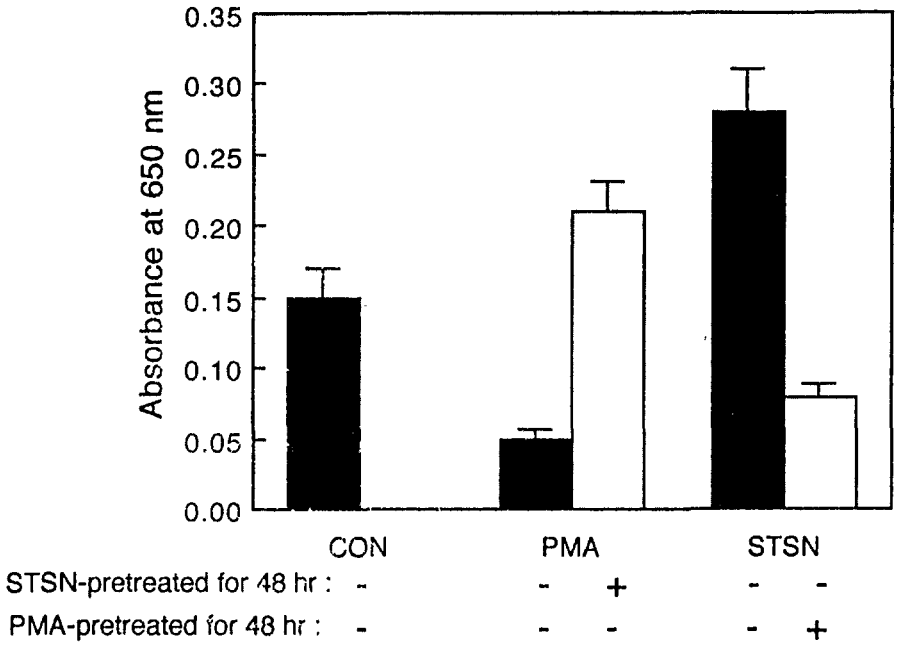


Fig. 6. Effects of PMA or STSN on chondrogenesis in STSN-pretreated or PMA-pretreated chondroblast. The cultures were supplemented with PMA (100nM) or STSN (20nM) for 48hr and changed with STSN- or PMA-containing media, respectively, throughout culture period and assayed at day 4 as described in Materials and Methods Values are the means SD of three experiments

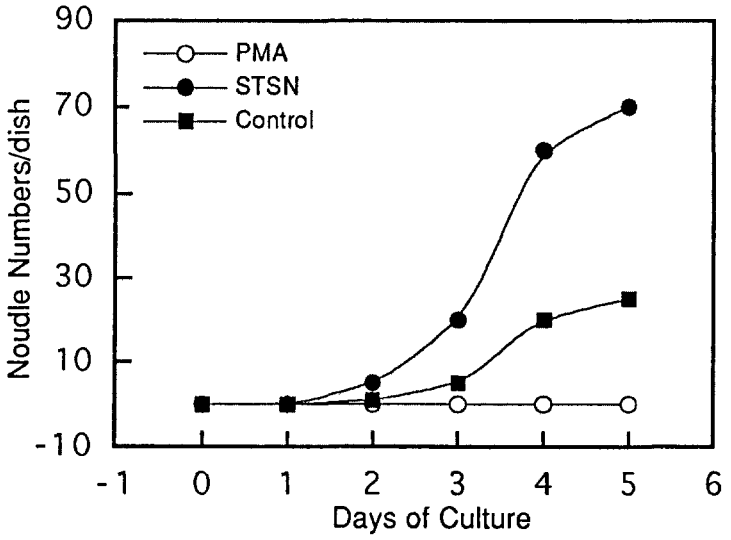


Fig. 7. Quantitation of chondrogenesis of micromass cultures occupied by cartilage as a function of time using human limb bud (Carnegie stage 16/17). Developing limb bud cells were cultured for 5 days. At indicated time points, the cells were fixed and stained and then the number of Alcian blue-staining nodules was determined microscopically.

centage of cells in S phase was relatively higher than that of cells in G_0/G_1 or G_2/M phaes. However, after 3 days, there was an increase in the proportion of cells arrested in G_0/G_1 phases, together with a significant decrease in the proportion of cells in the S phase; while the percentage of cells in G_2/M was not significantly affected.

PKC acts as A Negative Signal during Chondrogenesis

Increasing evidences demonstrated that PKC has a crucial role in the promotion of cellular differentiation including the regulation of chondrogenesis in chicken limb bud mesenchymal cells. We examined the effects of two PKC activity modulators on chondrogenesis in human cells. Treatment with PMA, a PKC activator, completely inhibited the chondrogenesis in a dose dependent manner (Fig. 5A). The optimal effect of PMA was shown at 200 nM of PMA. Concentrations of less than 2 nM were considerably less effective, in some cases, even ineffective. The inhibition of chondrogenesis by PMA fit with the range of concentration required to activate PKC by PMA. The biological inactive isomer, α-PDD, had no effect on the chondrogenesis in micromass culture. On the other hand, as seen in Figure 3B, STSN (which possess PKC-inhibiting properties) promoted chondrogenesis in a dose-dependent manner, indicating that PKC acts as a negative signal during chondrogenesis in human limb bud.

The promoting effect of STSN on chondrogenesis was markedly inhibited by prior treatment of cells with PMA for 48 h. In addition, PMA exerted no inhibitory effect on chondrogenesis in cells pretreated with STSN for 48 h (Fig. 6,7). These

results suggest that chondrogenic events might be settled at an early step *in vitro* and further demonstrate that, after initiation of differentiation, the events might not be overcome by antagonistic signal *in vitro*.

DISSCUSSION

The human embryonic mandible is formed from ectomesenchymal cells which originally migrate from the ectodermal neural crest into mesenchymal cells, later to be differentiated into Meckel's cartilage[28]. The mechanisms that lead to cellular differentiation in a spatially defined pattern in the mandible are not known.

Our results indicate that cells isolated from the limb bud mesenchyme showed significantly more differentiation than the cells isolated from the mandible (stage 16-17). It was rarely differentiated in the mandible. The tissues used presumably are quite heterogeneous in cell type which complicates the interpretation of the data since it is expected that a variety of cell types are present and that relative amounts of these different cell types varies considerably during the period studied. Our results suggest either that the majority of ectomesenchymal cells present in the early mandibular arch are destined to produce non-chondrogenic tissue and/or that only a small proportion of the cells possess a specific competence for chondrogenic expression that can be elicited in micromass cultures.

The differences in results may be understood due to: various species, differences in methods for determining embryo length; the fresh and intact condition of the embryo at the time of measurement; and the heterogenecity and multipotentiality in the cells' ability to differentiate bone, cartilage and the dermis of mandibular arch[10,13].

We examined the stage-related chondrogenic capacity of human mandibular and limb bud mesenchymal cells *in vitro* and report that limb bud mesenchymal cells as early as stage 16 can form cartilage in macromass cultures. Micromass cultures have been used to assess the differentiation potential of the mesenchymal cells in the developing limb bud[1,2,6 8,23,24]. The sequence of events leading to chondrogenesis by human limb bud is similar to that of chicken limb bud mesenchyme of comparable developmental stages as reported by others[23,24]. However the timing of human limb bud chondrogenesis reported here is different from that of a chicken's, in which the cartilage is first detected after 48 h of cultures[23,24]. In our results, aggregates are formed in the first 48 h of cultures and stainable cartilage matrix is detected in the following 24-48 h of cultures. These differences may be due to minor technical details and the culture conditions utilized, as we used the micromass culture techniques essentially those previously developed for the study of limb bud cells from chick embryos[23,24]. Aggregates of cells initially appear in the centers of the cultures and then differentiate into discrete cartilage nodules that gradually expand in the area to link up and form larger masses of cartilage over the next days in culture.

The *in vitro* stage-related chondrogenic potential of human limb bud was correlated with the *in vivo* expression of chondrogenic cells as determined by Alcian blue staining. In the developing human embryonic limb bud, a central core of mesenchymal cells began to aggregate at around stage 16-17, resulting in an almost 50% increase in cell-packing density. In this condensed core, Alcian blue positive cells were identified, which might secrete large amounts of cartilage-specific macromolecules such as type II collagen and sulfated proteoglycans.

During chondrogenesis, cell proliferation was observed in the first 3 days of culture and then decreased. This result was correlated with the cell cycle analysis which demonstrates that the percentage of cells in S phase was relatively higher than that of the cells in G_0/G_1 or G_2/M phases in the first 3 days of culture. However, after initiation of differentiation, the proportion of cells arrested in G_0/G_1 phases increased along with a significant decrease in the proportion of cells in the S phase, while the percentage of cells in G_2/M was not significantly affected. This result was, for the most part, in agreement with the pattern of cell proliferation during chondrogenesis of chicken limb bud mesenchymal cells[11].

Calcium and phospholipid-dependent PKC are thought to play an important role in the regulation of cell proliferation. PKC, the major cellular receptor for

phorbol esters, is thought to play a pivotal role in signal transduction by coordinating receptor-mediated hydrolysis of phosphorylation and the induction of gene expression[17]. Concomitant with the activation of PKC, a translocation of the enzyme takes place from the cytosol to the membrane, especially to the plasma membrane. PKC induces phosphorylation of a variety of proteins. Differential phosphorylation patterns induced by phorbol ester treatment have been observed in a previous study[26]. The basis of this difference in the effect of PKC activators on protein phosphorylation is not clear but it could involve differential regulation of PKC isotypes with different substrate specificities[19].

The involvement of PKC in the chondrogenesis of chicken limb bud has been well documented[11,27]. PKC activation, whether induced by receptor mediated phosphatidyl inositol metabolism leading to diacylglycerol production or by exogenous substances such as phorbol esters, occurs as an early event in many diverse cellular processes[19]. In this study, PMA, a potent activator of PKC, was found to inhibit chondrogenesis, whereas STSN, a PKC inhibitor, promoted the chondrogenesis in a dose-dependent manner. The inhibitory effect of PMA on chondrogenesis was not overcome by the sequential treatment with STSN. Moreover, PMA exerted no effect on chondrogenic inhibition prior to treatment with STSN. These results indicate that the chondrogenic events might be settled at an early step *in vitro* chondrogenesis and PKC may act as a negative modulator in limb bud mesenchymal cells of humans.

In conclusion, the present study defines stage-related chondrogenesis of human limb bud mesenchymal cells and further demonstrates that the chondrogenic event might be settled at an early step *in vitro* in chondrogenesis. Chondrogenesis of mesenchymal cells depends on a complex interplay between progenitor cells and other cells present in the limb bud microenvironment. Although many soluble factors secreted by these cells are involved in normal differentiation, a degree of cell aggregation, timing, and the extent of the activation of intracellular PKC might play key roles in limb bud chondrogenesis in addition to mandibular chondrogenesis of humans.

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