

Adhesion Molecules in Skeletogenesis: II. Neural Cell Adhesion Molecules Mediate Precartilaginous Mesenchymal Condensations and Enhance Chondrogenesis

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Neural cell adhesion molecules (NCAM) was expressed transiently by mesenchymal cells in precartilaginous condensations of the embryonic chicken limb but was lost upon differentiation into cartilage. Consequently, NCAM was present in the periphery of the limb anlagen but was absent in the cartilaginous center of the growing limb. To determine NCAM function in limb bud chondrogenesis we incubated dissociated stage 22/23 distal mesenchymal limb bud cells with Fab' fragments of antibodies to NCAM. Cell aggregation was inhibited by incubating the cells with anti-NCAM Fab'. These results suggest that NCAM may mediate the formation of precartilaginous condensations. This hypothesis was further tested using micromass cultures. NCAM expression in micromass cultures *in vitro* recapitulated that *in vivo*. NCAM was enriched in condensations of 2 day cultures, but was diminished and concentrically distributed around cartilage nodules in 4 day cultures. Anti-NCAM Fab' fragments reduced the area occupied by precartilaginous condensations and the degree of chondrogenic differentiation. Control antibody against chicken embryo fibroblasts had no effect. The effect of overexpressing NCAM was analyzed by electroporating expression vectors directing the synthesis of chicken NCAM. Limb bud cells cultured after electroporation with an NCAM expression vector displayed larger cartilage nodules and greater chondrogenic differentiation than cells electroporated with vector alone. The expression of NCAM in electroporated cells also increased. Control experiments using plasmids encoding β -galactosidase indicated that approximately 10% of the limb bud cells were transfected under these conditions. The results suggest that NCAM is involved in the chondrogenesis pathway by mediating the formation of precartilaginous condensations. © 1993 Wiley-Liss, Inc.

During the formation of the limb, the initial limb rudiment appears as the result of the interaction between mesoderm and ectoderm. Subsequent growth leads to the formation of the limb bud which itself diversifies into different mesenchymal tissues as well as receiving immigrating somatopleural cells and nerves. The result is a functional appendage with skeleton, muscle, tendon, ligament, and nerves organized in specific patterns (summarized in Hinchliffe and Johnson, 1980). The skeleton constitutes the structural framework of the limb. The location and pattern of the future limb skeleton is thought to be determined by the mesoderm. Initially, the limb mesenchyme is composed of uniformly and loosely packed cells. As the limb bud grows, the apical ectodermal ridge (AER) forms on the tip of the limb bud. Cells beneath the AER (100–200 μ m) form the progress zone where cartilage patterns are believed to be determined (Summerbell, 1974). Beginning at Hamburger and Hamilton's (1951) stage 23,

the cell density increases in the center of the limb buds to form the precartilaginous condensations (Thorogood and Hinchliffe, 1975). Gradually, the precartilaginous condensations are laid down from the proximal to distal limb. These precartilaginous condensations form the anlage of limb cartilages. Later endochondral ossification will replace most of these cartilage anlage into bone.

Two major processes are involved in the above limb chondrogenesis process: chondrogenic differentiation and pattern formation. The progression of chondrogenic differentiation can be followed by measuring the expression of markers of cytodifferentiation. Early mes-

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enchyme and precartilaginous condensations express type I collagen. The next phase of cartilage differentiation involves the enhanced expression of type II collagen, proteoglycans, link proteins, cartilage matrix proteins, etc. which form the cartilage matrix (Nah et al., 1988; Kosher et al., 1986). During this process, many molecules functioning as growth factors, growth factor receptors, extracellular matrix molecules, adhesion molecules, etc. are expressed in specific temporal and spatial distributions that are required for the transformation of a mesenchymal cell into a chondrocyte (reviewed in Tabin, 1991).

Recently, a group of molecules, the homeobox (Hox) genes, have been characterized (reviewed in McGinnis and Krumlauf, 1992) which appear to determine the pattern of the skeleton. The ordered expression pattern of Hox genes has been shown along the anterior-posterior axis (Hox 4 complex) (Nohno et al., 1991; Izpisua-Belmonte et al., 1991) and proximal-distal axis (Hox 1 complex) (Yokouchi et al., 1991) of the limb bud. Alteration of Hox genes by ectopic gene expression (Kessel et al., 1990), retinoic acid gradient, or null mutation (Le Mouellie et al., 1992) led to homeotic transformation of vertebrae and limb digits. If Hox genes are indeed regulators of cartilage pattern, they must be able to modulate the location, shape, and size of cartilage anlagen. This will mean that the influence on skeletal pattern has to be exerted on precartilaginous condensations.

It has been shown that fibronectin is one of the molecules that mediate the formation of precartilaginous condensations (Frenz et al., 1989a,b). It also has been suggested that hyaluronidase may enhance mesenchymal condensation by digesting hyaluronic acid and reducing the intercellular matrix (Kulyk and Kosher, 1987; Knudson and Toole, 1985; Singley and Solursh, 1981). However, the molecular basis of this process is still far from completely understood. We do not know how the condensation process is initiated, how the boundary is set, and how the shape is regulated. To study this process, it is critical to first characterize key molecules involved, particularly those involved in cell-cell interactions.

We have been studying the roles of neural cell adhesion molecules (NCAM) (reviewed in Edelman, 1992; Rutishauser et al., 1988) and became interested in precartilaginous condensations because we observed the transient expression of NCAM in limb precartilaginous condensations as well as in feather dermal condensations (Chuong and Edelman, 1985), muscle (Soler and Knudsen, 1991), and kidney tubule mesenchymal condensations (Crossin et al., 1985). Subsequent work has shown that NCAM is a mesenchymal cell adhesion molecule used in a variety of mesenchymal morphogenesis processes (reviewed in Chuong, 1990). Besides those mentioned, NCAM is also involved in myoblast adhesion (Dickson et al., 1990) and most recently, found to be transiently expressed in osteoblasts during both endochondral and intramembranous ossification (Lee and Chuong, 1992). During wound healing in the adult, NCAM is re-expressed transiently in the granulation tissue of wounded skin and in the blastema after bone fracture (Chuong and Chen, 1991).

In this study, we evaluated the physiological roles of NCAM in precartilaginous condensations. We showed

that NCAM is involved in limb bud cell aggregation. We altered NCAM activity in complementary ways to ascertain its function in limb chondrogenesis *in vitro*: first we suppressed NCAM activity by antibodies, then we increased NCAM expression by electroporating expression vectors encoding NCAM. The results showed that NCAM mediates the formation of precartilaginous condensation and enhances chondrogenic differentiation. Together with our recent finding that activin A enhances precartilaginous condensations and chondrogenic differentiation concomitant with the increase of NCAM (Jiang et al., 1993), the possible roles of NCAM in the molecular cascade of limb chondrogenesis are discussed.

MATERIALS AND METHODS

Materials

Fertilized white Leghorn chicken eggs were obtained from K and R Farms (Westminster, CA). Chicken embryos were staged according to Hamburger and Hamilton (1951). Antibody to NCAM was prepared as described in Chuong and Edelman (1985). Antibody to tenascin M1B4 was kindly provided by Chiquet and Fambrough (1984) through the Developmental Studies Hybridoma Bank (John Hopkins University, Baltimore, MD). Biotinylated peanut agglutinin and Texas red avidin were from Vector Laboratories (Burlington, CA). HNK is from the American Type Culture Collection. The monoclonal antibody to chicken type II collagen (2B1) was supplied by Dr. Richard Mayne, Hybridoma Core Facility of the Multipurpose Arthritis Center, University of Alabama at Birmingham.

Immunocytochemistry

Immunostaining was prepared as described (Jiang and Chuong, 1992). Briefly, the micromass cultures were fixed in 2.5% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and washed with PBS five times. Cultures were then incubated with 5% normal goat serum (NGS) in PBS for 1 hr, followed by primary antibodies dissolved in 5% NGS-PBS overnight. After washing five times, cultures were blocked by NGS and incubated with secondary antibodies which may be fluorescein-conjugated goat anti-rabbit (1:50) or biotinylated horse anti-mouse IgG followed by Texas red avidin D (1:100). Nonimmune rabbit IgG and P3U monoclonal ascites have been used as negative controls.

Limb bud cell aggregation assay

Hamburger and Hamilton stage 22-23 chicken embryos were used. The distal one third of the limb bud was dissected and the epithelium was removed. Limb bud mesoderm was pooled and digested with 0.1% trypsin and 0.1% collagenase (Worthington Corp., Freehold, NJ) in calcium and magnesium free media at 37°C for 10 min. The digestion was stopped by the addition of fetal calf serum (FCS) and the tissues were gently triturated. The resulting cell suspension was washed with ice cold Dulbecco's modified Eagle's media (DMEM) and centrifuged at 224g for 3 min at 4°C. The number of limb bud cells was counted with a hemocytometer. 3×10^5 cells in 0.6 ml were then shaken in a 24 well dish precoated with elastomere to prevent cell

TABLE 1. Anti-NCAM inhibits precartilaginous condensations and chondrogenesis

Experiment (no.)	Incidence (%) of the size of precartilaginous condensations ¹				Incidence (%) of the size of chondrogenic regions ²			
	<30	30-60	60-90	>90	<30	30-60	60-90	>90
Media (>10)	0	0	0	100	0	0	0	100
Nonimmune (>10)	0	0	0	100	0	0	0	100
R 500 (2)	0	0	0	100	0	0	0	100
R α NCAM-1								
16 μ g/ml (5)	0	0	66	33	0	20	80	0
80 μ g/ml (7)	0	0	75	25	0	57	28	14
200 μ g/ml (4)	50	25	25	0	75	0	25	0
R α NCAM-2								
200 μ g/ml (5)	20	80	0	0	20	80	0	0
HNK ³								
200 μ g/ml (2)	0	0	0	100	0	0	0	100

¹Compared to that of control, which is set as 100.

²Compared to that of control, which is set as 100.

³HNK recognizes a carbohydrate epitope on NCAM.

binding to the plastic dish. Shaking was at 70 rpm in a 37°C humidified incubator. Aliquots (100 μ l) were removed at 0, 20, 40, or 60 mins and fixed in 900 μ l of fixative (2.5% paraformaldehyde in PBS). The number of particles was then measured on a Coulter Counter (Coulter Electronics, Hialeah, FL). The settings were Attenuation = 2, Aperture = 8, Threshold = 20. Duplicate experiments were carried out for each sample.

Micromass cultures

Micromass cultures were prepared according to Ahrens et al. (1977), with some modification. Cells were prepared as for the limb aggregation assay described above. 3×10^5 cells in 15 μ l were then plated on a 35 mm tissue culture dish (Falcon, Becton Dickinson, Lincoln Park, NJ) and the drop was left for cellular attachment for 2 hr in a 37°C humidified incubator. Following that, the dish was gently flooded with 1.5 ml culture medium and cultured at 37°C and 5% CO₂/95% air. Cells were cultured in either serum free defined medium composed of 60% Ham's F12 nutrient mixture/40% DMEM, 5 μ g/ml insulin, 5 μ g/ml transferrin, 50 μ g/ml L-ascorbic acid, 100 nM hydrocortisone (Paulsen et al., 1988), or DMEM containing 2% fetal bovine serum. Duplicate samples were always used for each experiment.

Quantitation of area of precartilaginous condensations and degree of chondrogenesis

Micromass cultures were first observed under phase contrast inverted microscopy and photographs were taken at the indicated times for image analyses. Cultures were fixed with 2.5% paraformaldehyde and washed by PBS. Alcian blue staining was done by using 1 mg/ml alcian blue dye (Sigma, St. Louis, MO) in 0.1 N HCl (pH 1). After overnight staining, the cultures were destained with 70% ethanol (Lev and Spicer, 1964). The area occupied by precartilaginous condensations and the area stained by alcian blue were quantitated separately with Sigma Scan measurement software (Jandel Scientific, Sausalito, CA) with a Summagraphics digitizer pad (Fairfield, CT). These measurements were divided by the total area occupied by the micromass cultures and shown as the percentage of control cultures (Table 1). Chondrogenesis was also quantified by extracting the alcian blue dye from each micromass cul-

ture with 0.5 ml 4 M guanidine HCl, pH 5.8. The extract was read at OD 600 nm (Hassell and Horgan, 1982). Large aggregates are defined as cell groups larger than 350 μ m in diameter.

Antibody perturbation

Rabbit IgG were purified by DEAE column chromatography and Fab' fragments were generated according to Brackenbury et al. (1977). For mouse monoclonal antibodies, mouse IgG were purified from protein G affinity membrane disk (Gibco/BRL, Grand Island, NY) and digested with papain (10 μ g papain/mg IgG) following the method of Harlow and Lane (1988).

Electroporation of plasmids into primary limb bud cells

The cells were pelleted and resuspended in culture medium and plated for micromass cultures. Alternatively, cells for electroporation studies were suspended on ice in 400 μ l of DMEM containing 5 μ g plasmid DNA and 250 μ g/ml salmon sperm DNA and electroporated on a Gene Pulser (BioRad, Richmond, CA) at 0.7 V, 960 μ Fd. The cells used for the electroporation studies had a survival of 50-60% after electroporation as judged by the exclusion of trypan blue. The cells were then pelleted and resuspended in culture medium to a density of 2×10^7 cells/ml. Fifteen microliters of this cell suspension was plated on 12 mm Millicell-CM culture plate inserts with 0.4 μ m pore size (PICM01250, Millipore, Bedford, MA).

The expression vector pCH110, encoding β -galactosidase is under the transcriptional regulation of the SV40 promoter and transcribed mRNAs are stabilized by the SV40 polyadenylation signal (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The 140 KD form of NCAM (pEC1402) was subcloned after the SV40 promoter of pCH110 and is under similar transcriptional and polyadenylation control (Edelman et al., 1987). While pEC1402 contains the 3' end of pCH110, it no longer expresses β -galactosidase.

β -galactosidase activity was detected by fixing the micromass cultures in 2.5% paraformaldehyde, pH 7.0, and incubating the cultures in 0.5 mg/ml X-gal in 50 mM phosphate buffer containing 5 mM potassium ferri-

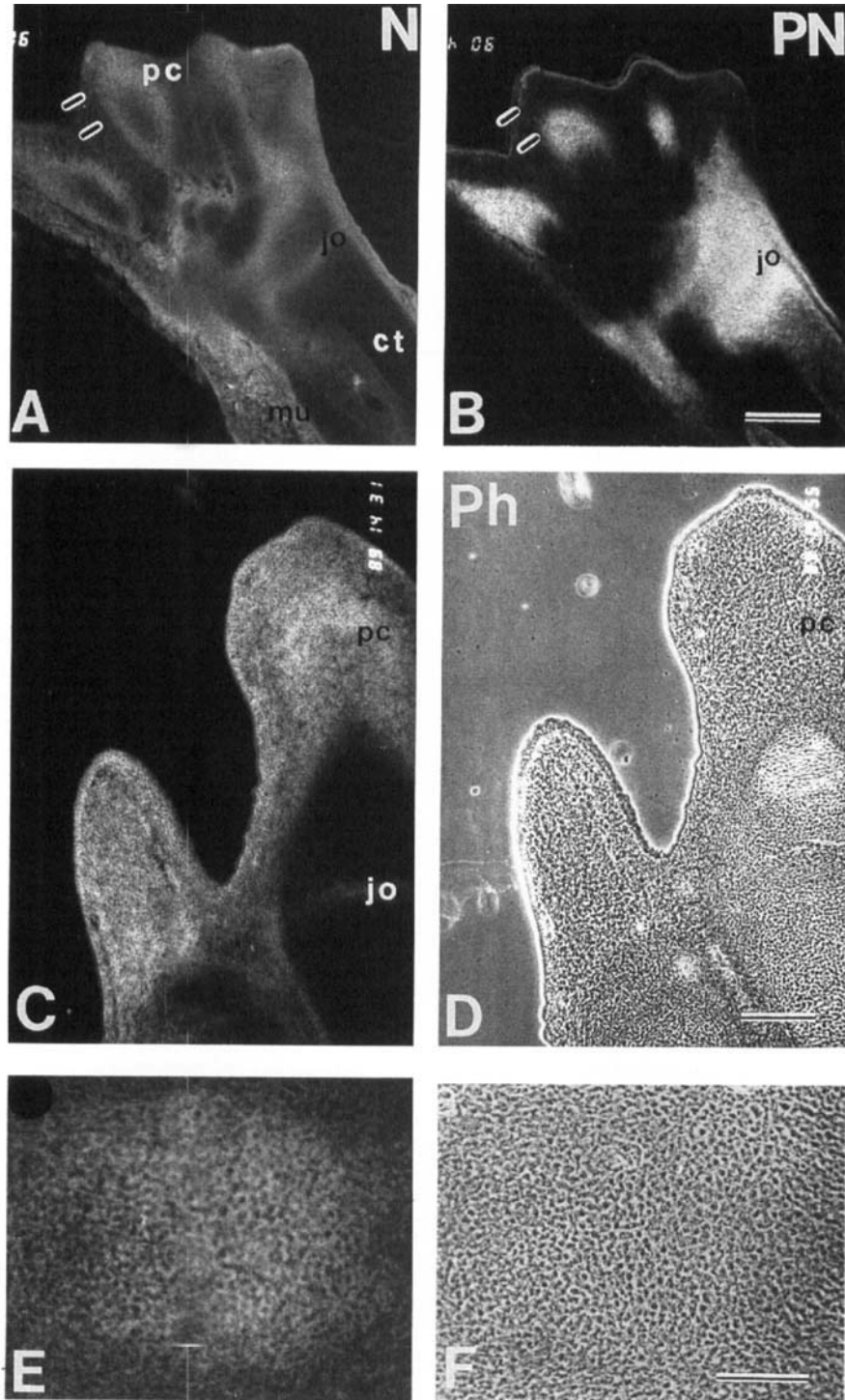


Fig. 1. Expression of NCAM in precartilaginous condensations of developing limb bud. Longitudinal sections of stage 27 (A, B) and stage 30 (C, D) chicken hindlimb buds and cross sections of stage 30 distal limb buds (E, F) were stained with antibodies to NCAM (A, C, E) and peanut agglutinin (B). NCAM is present in the condensing mesenchyma of the developing limb anlage. The presence of NCAM in the round and well packed cells inside the condensation and the absence of NCAM in the loosely arranged and elongated cells can be appreciated in panels E and F. In regions with overt chondrogenic

differentiation, NCAM staining disappeared (A, C). Double staining of the cartilage blastema with peanut agglutinin showed that the expression of NCAM is distal to that of peanut agglutinin binding molecules. The two bars in panels A and B marked the same positions. ct, differentiated cartilage; jo, joint; mu, muscle; N, NCAM; pc, precartilaginous condensation; Ph, phase; PN, peanut agglutinin. Paired micrographs (A + B; C + D; E + F) were taken at the same magnification. Bars: (B) 500 μm ; (D) 200 μm ; (F) 200 μm .

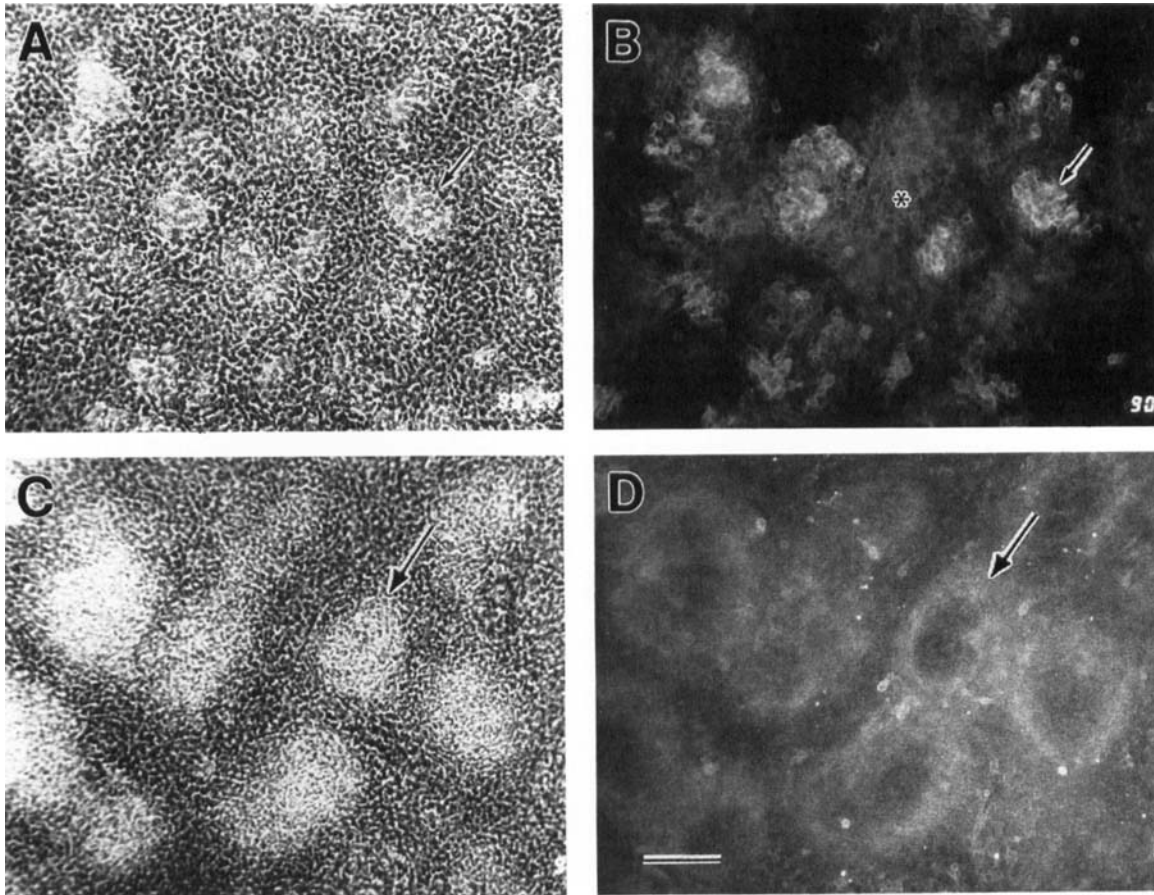


Fig. 2. Expression of NCAM in precartilaginous condensations in micromass cultures *in vitro*. Stage 23 distal limb bud cells were prepared for micromass cultures and grown in culture for 1.5 (A, B) or 4 days (C, D) before fixation and staining with anti-NCAM antibodies. Photomicrographs showing phase contrast (A, C) and fluorescent (B, D) images of the same fields are presented. A, B: At day 1.5 in culture, the small cell aggregates were enriched with NCAM (small arrows).

Cells adjacent to these aggregates were moderately positive for NCAM (regions with asterisk). Cells outside the aggregate zone were negative for NCAM. C, D: At day 4, the precartilaginous condensations have become cartilage nodules (big arrows). As cartilage nodules mature, the centers became NCAM negative. NCAM staining remained on the periphery of the nodules but was diminished in intensity. Bar: (D) 200 μ m.

cyanate and 5 mM potassium ferrocyanate (Sanes et al., 1986). A blue color indicated the presence of β -galactosidase.

RESULTS

NCAM is present in precartilaginous condensations *in vivo* and in micromass cultures

To determine the role of NCAM in cartilage development, stage 27 and 30 limb buds were sectioned and immunologically stained. NCAM was present in the condensing mesenchyme of the cartilage anlage including those at the distal end, which are involved in cartilage elongation, and those in the periphery, which are involved in increasing cartilage diameter (Fig. 1A,C). As cartilage became more differentiated in the more proximal locations, NCAM disappeared from chondroblasts but remained present in the perichondrium (Fig. 1A,C). The distal end where the digit anlage continues to grow in length remained NCAM positive. The enrichment of NCAM in the condensing mesenchyme vs. the surrounding mesenchymal cells can be seen clearly in the cross sections of the precartilaginous condensa-

tion (Fig. 1E,F). Peanut agglutinin has been reported to bind to the cartilage blastema (Aulhouse and Solursh, 1987; Zimmerman and Thies, 1984). In comparison using double staining, the presence of NCAM was distal to the region stained by peanut agglutinin, and hence NCAM expression in precartilaginous condensations appears to precede the molecules recognized by peanut agglutinin.

To explore the roles of NCAM in micromass cultures (Ahrens et al., 1977), we examined the expression of NCAM in these cultures. Within 1.5 days, the homogeneously distributed limb bud cells formed aggregates which represent precartilaginous condensations. In Figure 2A and B, these condensations can be seen to be enriched for NCAM (arrows). Surrounding the condensations are a zone of moderately NCAM positive cells (asterisks) which are cells which will probably be recruited into the precartilaginous condensations. At day 4 in culture, the precartilaginous condensations have differentiated to cartilage nodules. NCAM staining disappeared from the center of these cartilage nodules but remained in the periphery (Fig. 2C,D). The expression

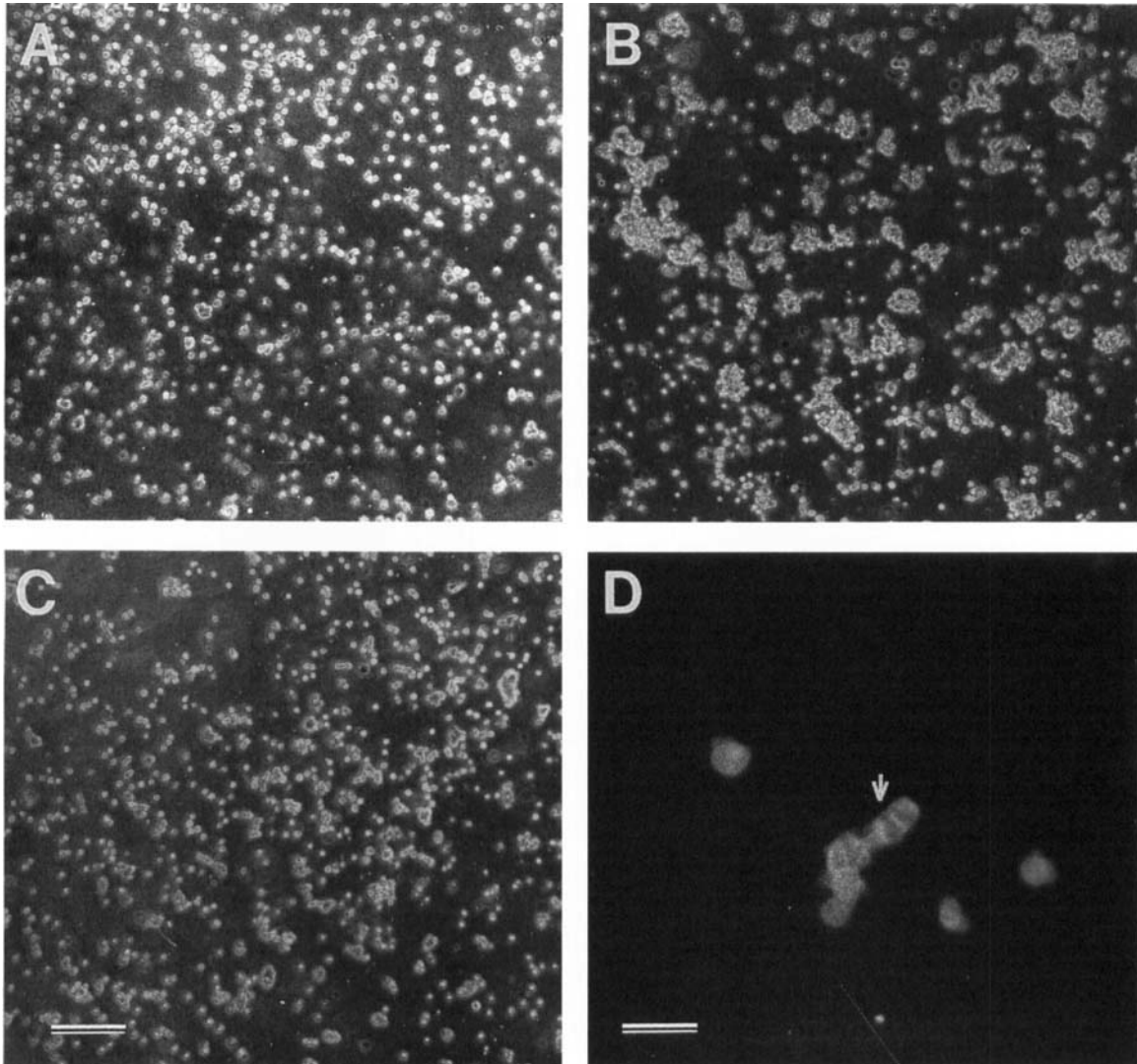


Fig. 3. Inhibition of limb bud cell aggregation by anti-NCAM antibodies. Stage 23 distal limb bud cells were prepared as a single cell suspension and shaken for 60 min at 37°C. **A:** Time 0. **B:** 60 min in the presence of 400 µg/ml nonimmune Fab'. **C:** 60 min in the presence of 400 µg/ml anti-NCAM Fab'. Note in B, many cell aggregates of 10–50 cells formed. With anti-NCAM, some cell aggregates formed, but few were larger than ten cells. For quantitative measurement, see Figure

4. **D:** Immunofluorescent staining of limb bud cells 90 min after shaking. Note the intensified NCAM staining at the cellular interface (arrow) within the aggregate. The three single cells had weak and diffuse NCAM immunoreactivity within the cytoplasm. Micrographs shown in panels A–C were taken at the same magnification. Bars: (C) 200 µm; (D) 50 µm.

pattern of NCAM in the chondrogenic process in vitro parallels that found in vivo (compare Fig. 2B,D and Fig. 1A,E).

We then tested the functional roles of NCAM in pre-cartilaginous condensations using limb bud cell aggregation assays and micromass cultures.

Anti-NCAM Fab' inhibited limb bud cell aggregation

We first determined whether NCAM is involved in the initial aggregation of dissociated limb bud cells. Single cells were prepared from stage 23 distal limb buds (Fig. 3A). After 60 min of shaking at 70 rpm at 37°C, many aggregates of about 10–50 cells formed

even in the presence of nonimmune Fab' fragments, or Fab' fragments from antibodies raised against chicken embryonic fibroblasts (Fig. 3B). In the presence of anti-NCAM Fab' (400 µg/ml), both the number and size of the aggregates were drastically reduced, and the aggregates appeared to be composed of fewer cells, in the range of five to ten cells (Fig. 3C). Quantitative measurement of the particle numbers with a Coulter counter allowed us to monitor the progress of aggregation. Shown in Figure 4 is one example of the five independent cell aggregation assays we performed. In every experiment, anti-NCAM inhibited aggregation by about 50–60%. In the experiment shown in Figure 4, anti-chicken embryonic fibroblasts Fab' (R 500) had no significant effect on aggregation in comparison with

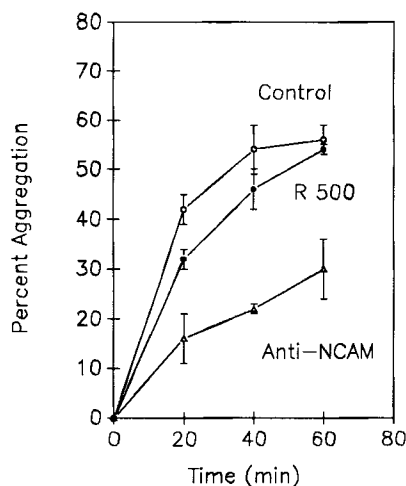


Fig. 4. Time course of the inhibition of limb bud cell aggregation by anti-NCAM antibodies. Single cell suspensions of limb bud cells prepared as in Figure 3 were shaken at 37°C in the presence of 400 µg/ml Fab' from nonimmune serum (open circles, Control), antifibroblast antiserum (closed circles, R 500), or anti-NCAM antiserum (open triangles). The number of particles was counted at 0, 20, 40, and 60 min. The percent aggregation was calculated for each group using the following formula: percent aggregation = $100 \times (1 - P_t/P_0)$, where P_0 is the number of particles at time 0, and P_t is the number of particles at the indicated times. In the presence of anti-NCAM, the aggregation was reduced.

media alone at the 40 and 60 min time points. Nonimmune IgG (Control) also did not inhibit aggregation.

The presence of NCAM in these limb bud cells was also examined. At time 0, some cells did not have NCAM immunoreactivity, while some cells had NCAM diffusely distributed in their cytoplasm, and some cells had NCAM all over the cell surface. As cell aggregates formed, NCAM became intensified on the cell surface, and particularly enriched at cell:cell boundaries within the aggregates (Fig. 3D, 90 min after shaking). These data suggest that NCAM is involved in limb bud cell aggregation, although cell aggregation is not exclusively dependent on NCAM since aggregates ultimately formed after an extended shaking time period.

The roles of NCAM in chondrogenesis were then tested by varying the level of NCAM activity in micromass cultures. Cultures were incubated with anti-NCAM Fab' to study the effect of suppressed NCAM activity. We also electroporated additional copies of NCAM genes into the micromass cultures for overexpression studies.

Anti-NCAM Fab' decreased the area of precartilaginous condensations and the degree of chondrogenesis

Micromass cells were cultured for 4 days in media containing nonimmune Fab', antifibroblast Fab' (R 500), or anti-NCAM Fab' in serum free defined media (Fig. 5). Both the area occupied by condensations and the degree of cartilage differentiation were reduced in the presence of anti-NCAM Fab' (Fig. 5). Similar results have been obtained when media contained 2% FCS. The transformation of condensations into cartilage nodules began from the center of the condensations

and can be judged by positive alcian blue staining. In controls, the cells that compose the cartilage nodules were tightly packed and the whole nodules were stained positive for alcian blue (Fig. 5A,B). With anti-NCAM, although cellular clusters did form, they appear to be less condensed and only cells in the center of the cluster exhibited alcian blue staining (Fig. 5C,D, black arrows pointed to chondrogenic regions, blank arrows pointed to nonchondrogenic regions). In 2 more days, these nonchondrogenic regions will chondrify. Therefore we termed these clusters at day 4 cultures precartilaginous condensations.

The results of micromass experiments are summarized in Table 1. The percent of the area occupied by precartilaginous condensations was determined by image analyses and expressed as the percent of the control. The degree of chondrogenic differentiation was quantified by extracting the alcian blue dye bound to the micromass cultures as well as the area occupied by alcian blue staining. Cartilage nodules formed in the presence of control nonimmune Fab' and antifibroblast Fab' fragments. With anti-NCAM Fab' fragments, the areas occupied by precartilaginous condensations and cartilage nodules were both reduced. The reducing effect of anti-NCAM Fab' fragments was dose dependent (Table 1). In addition, with the same dose of anti-NCAM Fab' fragments, there was a greater inhibitory effect on chondrogenesis than on precartilaginous condensations (Table 1, Fig. 6). Anti-NCAM Fab' fragments reduced the area occupied by precartilaginous condensations by about 50% and the area occupied by differentiated cartilage by about 68% (Fig. 6). Two independently raised rabbit anti-affinity purified chicken NCAM Fab's α NCAM-1 and α NCAM-2 had similar effects. The HNK monoclonal antibody, recognizing a carbohydrate epitope shared by NCAM, did not inhibit precartilaginous condensation formation nor chondrogenesis. The results implied that NCAM is involved in the formation of precartilaginous condensations, and the inhibition of NCAM leads to suppression of cytodifferentiation.

Overexpression of NCAM enhanced the formation of precartilaginous condensations and chondrogenesis

We then tested the role of NCAM in chondrogenesis by electroporating expression vectors directing the synthesis of the 140 KD form of NCAM (pEC1402) or β -galactosidase (pCH110) into limb bud cells. Both expression vectors used the SV40 promoter and the SV40 polyadenylation signal (Edelman et al., 1987). When X-gal staining was used to determine electroporation efficiency, approximately 10% of the limb bud cells electroporated with pCH110 expressed β -galactosidase. β -galactosidase was expressed only by cells electroporated with pCH110 and its distribution suggests that expression was not dependent on cartilage differentiation (Table 2). Incorporation of plasmid pEC1402 had profound effects on cell behavior resulting in big cell aggregates (>350 µm in diameter, with at least 1,000 cells in one aggregate) protruding out of the substrate (Fig. 7A,B). The effect was seen more clearly when Millipore filters were used as the substrate rather than tissue culture plastic. We have noted an increase in cell

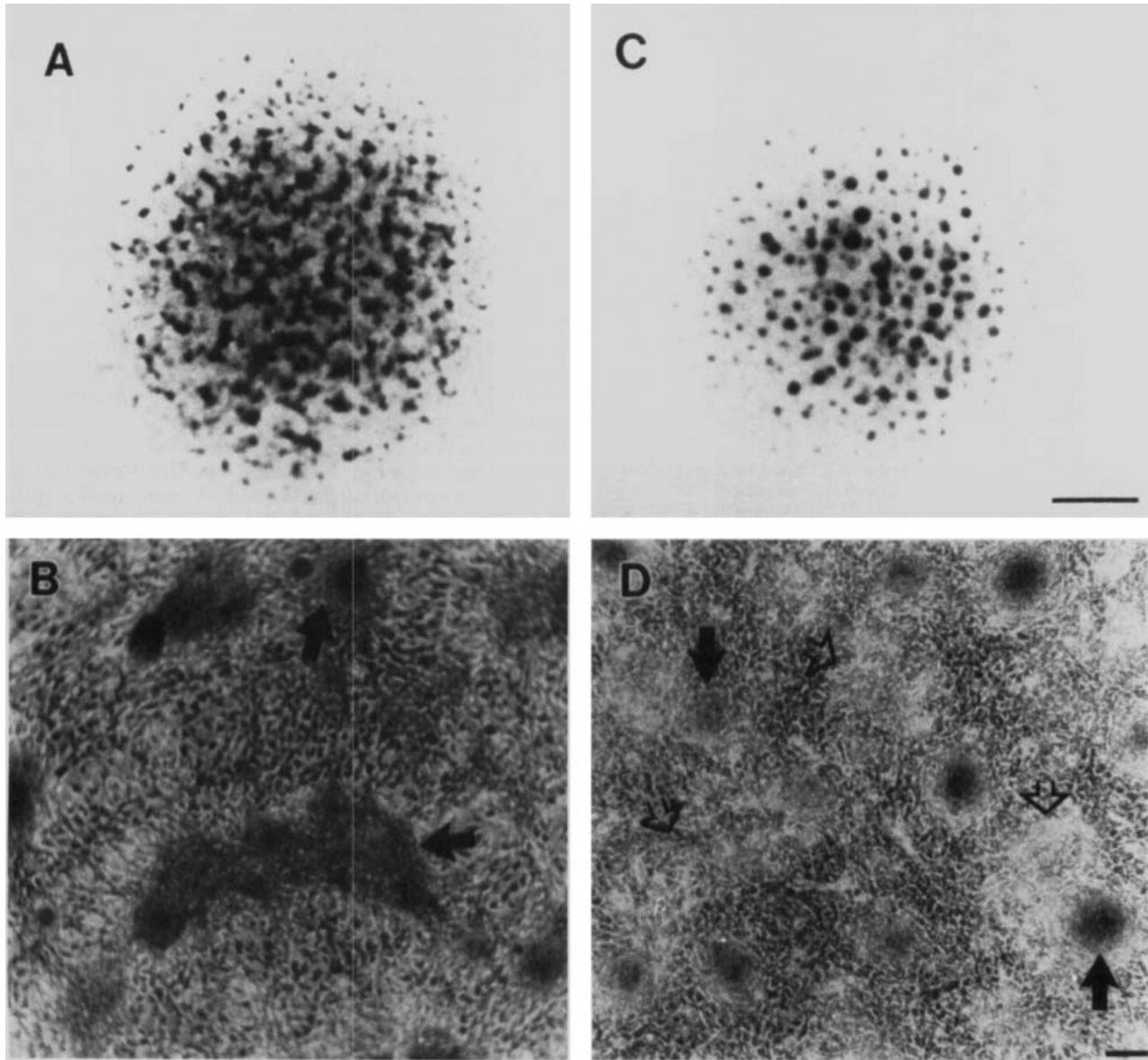


Fig. 5. Antibodies to NCAM inhibit chondrogenesis in micromass cultures. Micromass cultures grown for 4 days with 400 $\mu\text{g/ml}$ Fab' fragment antibodies from nonimmune serum (A, B) or antibodies against NCAM (C, D) were fixed and stained with alcian blue to detect the highly sulfated proteoglycan associated with cartilage differentia-

tion. The number and size of condensations as well as the degree of chondrogenetic differentiation were reduced. Black arrows point toward regions of chondrogenesis, while blank arrows indicate nonchondrogenic regions (C, D). B and D are higher magnification views of A and C, respectively. Bars: (A, C) 1 mm; (B, D) 100 μm .

motility on these Millipore filter inserts which may facilitate the formation of these big aggregates. The big aggregates were observed in nine out of 12 experiments (75%) using pEC1402 (Table 2).

These big aggregates formed cartilage nodules. When stained with antibody to collagen II, the whole nodules were brightly stained (Fig. 7D,H). The total amount of collagen II is higher than that of control. Tenascin, an extracellular matrix adhesion molecule present in chondroblasts (Mackie et al., 1987), is also increased in the pEC1402 cultures (Fig. 7C,G). The amount of NCAM in the aggregates is also greatly increased (Fig. 7B,F). Most interestingly, the weakly

NCAM positive cells observed in control micromass cultures (Fig. 2D, 7B) have disappeared (Fig. 7F), suggesting that they have been recruited to the larger aggregates.

DISCUSSION

We have been investigating the role of the cell adhesion molecules in embryonic skeletogenesis. Previously we have examined the function of cell adhesion molecules in osteogenesis (Lee and Chuong, 1992). In the present study we have examined the expression of NCAM during limb chondrogenesis and tested its function by modulating NCAM activity. The results showed

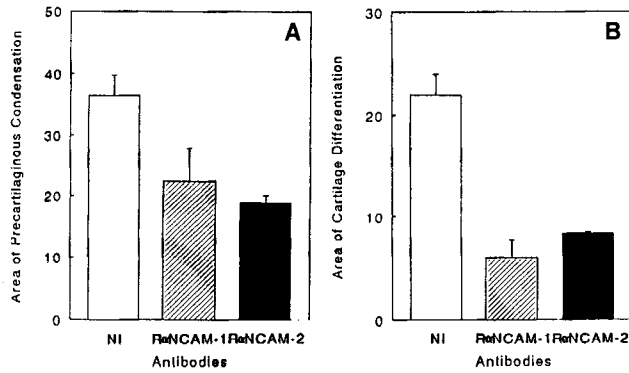


Fig. 6. Antibodies to NCAM decrease the size of precartilaginous condensations and cartilage nodules. Micromass cultures grown for 4 days in the presence of nonimmune rabbit serum or in the presence of two rabbit anti-NCAM Fab' fragments at 400 μ g/ml were fixed and stained for cartilage differentiation with alcian blue. **A:** The percent of the culture area occupied by precartilaginous condensations was decreased by 50% in the presence of anti-NCAM Fab'. **B:** The percent of the culture area occupied by cartilage nodules was reduced 65–70% by anti-NCAM Fab'.

that NCAM is present in precartilaginous condensations both in vivo and in micromass cultures in vitro, that NCAM is involved in the formation of precartilaginous condensations, and that NCAM can regulate the degree of chondrogenesis.

NCAM is an adhesion molecule that mediates precartilaginous condensations

During cartilage development, NCAM is present in the precartilaginous condensations either at the distal tip where cartilage increases in length or in the perichondral region where cartilage increases in diameter. The expression, however, is transient and highly dynamic. NCAM has also been implicated in the precartilaginous condensations of the talpid chicken. Talpid chickens form syndactylous and polydactylous limbs and have abnormally large precartilaginous condensations and increased limb bud cell adhesivity (Ede and Flint, 1975). We have shown that the expression of NCAM is increased in these talpid precartilaginous condensations and that anti-NCAM antisera can reduce chondrogenesis of micromass cultures derived from talpid limb buds (Chuong et al., 1992b and manuscript in preparation).

Inhibiting NCAM expression with anti-NCAM Fab' fragments reduces limb bud cell adhesion (Fig. 4) and

decreases the size of precartilaginous condensations in the micromass cultures in a dose dependent manner (Table 1). Although NCAM appears to be involved in the process of precartilaginous condensations, the partial response to anti-NCAM suggests that other adhesion molecules are also involved. Antifibronectin has been reported to reduce the size of precartilaginous condensations (Frenz et al., 1989b; Chuong et al., 1992b). Tenascin promotes chondrogenesis while antitenascin inhibits chondrogenesis. Antilaminin had no effect (Mackie et al., 1987; Chuong et al., 1992b). The role of integrins, receptors for fibronectin, has not been explored. We have proposed that NCAM and fibronectin enhance the condensation process by increasing cell-cell adhesion, while tenascin facilitates the condensation process by decreasing cell-substrate adhesion (Chuong et al., 1992b).

Cell adhesion molecules have also been implicated in the formation of other tissues arising from mesenchymal condensations. During skin morphogenesis, anti-NCAM Fab' fragments led to the uneven segregation and loss of dermal condensations, resulting in a distortion of the regular hexagonal feather pattern. Anti-integrin β 1 disrupted the epithelial-mesenchymal interactions and dramatically inhibited further dermal condensation. Antifibronectin had a similar but weaker effect than anti-integrin. Antitenascin led to cessation of feather bud growth (Jiang and Chuong, 1992). In muscle, NCAM transfected myoblasts have enhanced myogenesis (Dickson et al., 1990). These results suggest that NCAM is indeed a mesenchymal adhesion molecule. Microinjection of anti-NCAM into wounded amphibian limb blastema can perturb limb regeneration (Maier et al., 1986). Although the authors attributed this finding to inhibition of a neural contribution, it cannot be ruled out that anti-NCAM acted directly upon mesenchymal tissue morphogenesis.

Future research will be directed to elucidate the molecular pathways of these mesenchymal condensations.

NCAM and chondrogenic differentiation: Is NCAM a modulator or an essential component of the signal transduction pathway?

In cultures treated with anti-NCAM, chondrogenic differentiation decreased, while in those treated with NCAM plasmids, chondrogenic differentiation increased. These results do not differentiate between the following two possible mechanisms. One is that increasing NCAM expression in a subpopulation of cells increases the adhesiveness of these limb bud cells,

TABLE 2. Electroporation of plasmids containing NCAM genes increases the size of precartilaginous condensations and chondrogenic differentiation¹

Clone (no.)	Incidence of large aggregates	Nodule center regions				Molecular expression nodule periphery region				Internodule region			
		β G	N	TN	C	β G	N	TN	C	β G	N	TN	C
pCH110 (4)	7% (1/14)	+++	—	++	++	+++	+	++	+	+++	+	—	—
pEC1402 (4)	75% (8/12)	—	++	+++	+++	—	+++	+++	+++	—	—	+	—

¹Micromass cultures were electroporated with the indicated vectors and cultured for 4 days. The formed aggregates were analyzed by image analyses. Molecular expression was determined by immunofluorescent staining for β -galactosidase (β G), NCAM (N), tenascin (TN), and collagen II (C) in the cartilage nodule regions, the nodule periphery regions, and the internodule regions. The intensity of immunofluorescence was determined semiquantitatively. Due to the heterogeneity of the micromass cultures, the scoring was done in three different regions: nodule center, nodule periphery, and internodule regions. The intensity shown is the average of four independent experiments. Alcian blue staining was not used due to the high background binding in these culture inserts. Large aggregates are defined as cell groups larger than 350 μ m in diameter. The data shown are compiled from 14 different experiments (n). —, no staining; +, weak staining; ++, moderate staining; +++, strong staining.

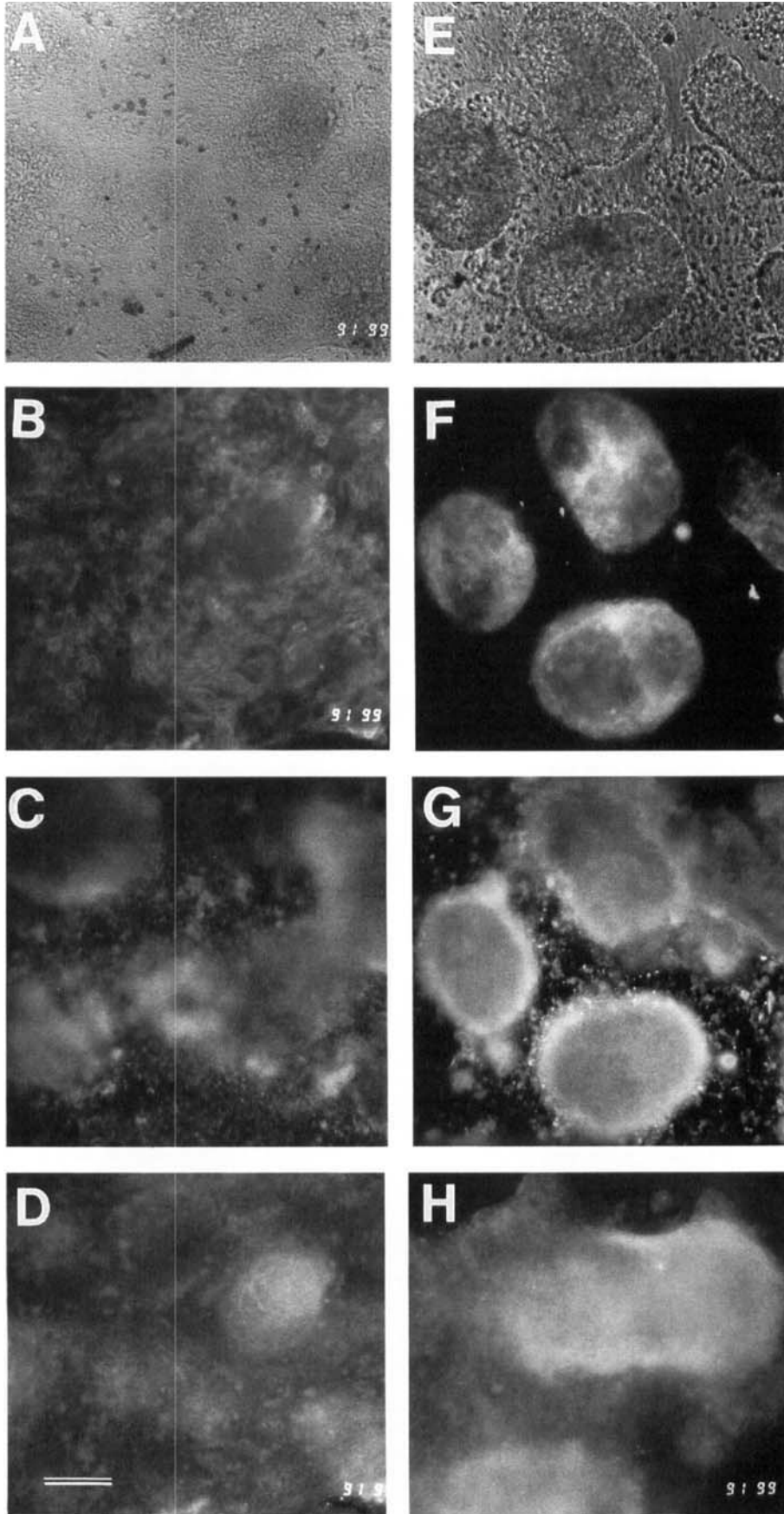


Fig. 7.

hence increasing the opportunities that these cells will end up within the precartilaginous condensations. Once recruited into the aggregate, the shape and intercellular interactions of a cell will be influenced by the localized microenvironment which may have profound effects on its differentiation pathway. In this model, the enhanced chondrogenic differentiation would be the result of normal maturation of larger condensations. A second mechanism is that NCAM is directly involved in the signal transduction of chondrogenic differentiation in limb bud cells.

Although we cannot completely rule out either of these possibilities at this time, several observations favor the first possibility. With higher concentrations of anti-NCAM Fab', chondrogenic differentiation is reduced, but not completely suppressed. This suggests that chondrogenesis can occur independent of the presence of NCAM (Table 1). In contrast, in the center of cartilage nodules where differentiation occurs and NCAM normally disappears, overexpression of NCAM did not seem to suppress cartilage differentiation (Table 2). Furthermore, the alteration of NCAM level appeared to be disproportionate to the degree of alteration of chondrogenic differentiation. In antibody perturbation experiments, the reduction of chondrogenesis appears to be more sensitive than the decrease of precartilaginous condensations (Table 1) and several condensations can be seen without alcian blue staining, a sign of chondrogenic differentiation (Fig. 5). In exogenous NCAM expression experiments, the increase of chondrogenesis was also much higher than the estimated 10% of cells overexpressing NCAM. Thus, NCAM may play a modulatory role rather than a direct role in the chondrogenic differentiation pathway. During neural morphogenesis similar regulatory roles played by NCAM have been proposed (Rutishauser et al., 1988).

Whether NCAM plays a direct signal transduction role can be further tested by electroporation of expression vectors containing NCAM with different cytoplasmic domains. If vectors with deleted cytoplasmic domains still promote chondrogenesis, it will demonstrate that only adhesion itself is necessary. This, however, does not downplay the physiological importance of NCAM. By bringing cell surfaces close together, NCAM allows specific chondrogenic signals to be transmitted. A similar two tier adhesion-signalling hypothesis has been proposed in lymphocyte adhesion in which a general adhesion molecule works as a scanning mechanism, while more specific interactions occur after-

wards (Lawrence and Springer, 1991). We propose that NCAM forms the boundary of cell groups to define the size and shape of precartilaginous condensations. Additional signals are required for chondrogenic differentiation to take place in all cells within the condensation. One example of such a molecular event may be gap junction communication. Gap junctions have been shown to be enriched in precartilaginous condensations (Coelho and Kosher, 1991) and dermal condensations (Serras et al., in press), and anti-NCAM has been shown to inhibit subsequent gap junction formation and differentiation in neural plate formation (Keane et al., 1988).

Peptide growth factors and Hox genes can regulate, either directly or indirectly, the expression of NCAM in limb chondrogenesis

Peptide growth factors of the transforming growth factor (TGF) β superfamily and Hox containing transcription factors have recently been characterized and appear to have profound effects on the differentiation and pattern determination of skeletal formation. As NCAM mediates precartilaginous condensations, we wonder what factors could regulate NCAM gene expression. It is possible that NCAM may be regulated by these two families of molecules and may mediate the downstream effects of these molecules. TGF β and several bone morphogenetic proteins can induce chondrogenesis (Wozney et al., 1988; Joyce et al., 1990), and TGF β can stimulate precartilaginous condensations and fibronectin expression (Leonard et al., 1991). Recently, we found that activin can increase NCAM expression while stimulating the growth of precartilaginous condensations (Jiang et al., 1993). Thus, some of these peptide growth factors may have their effects mediated by adhesion molecules.

Another connection comes from the recent progress in Hox genes. Hox proteins are a group of homologous DNA binding proteins which act as transcription factors (reviewed in Goulding and Gruss, 1989). Transgenic mice ectopically expressing Hox-1.1 have craniofacial and vertebral abnormalities (Balling et al., 1989; Kessel et al., 1990). Also, transgenic mice with targeted gene disruption of Hox 1.5 have craniofacial abnormalities similar to those found in human DiGeorge's syndrome (Chisaka and Capecchi, 1991) while disruption of Hox 3.1 leads to alterations of the vertebral column (Le Mouellic et al., 1992). These studies have led to the hypothesis that certain combination of Hox genes (Hox code) determine the pattern of limb and axial skeleton. How can the pattern determining effect of Hox genes be exerted? It has to be through modulating the morphology of precartilaginous condensations. The current work showed that NCAM has a powerful effect in modulating chondrogenesis and is one of the likely downstream candidates of Hox genes. The discovery that Hox gene binding sites are present in the 5' region of NCAM genes and that the expression of transfected Hox genes in cell lines alters the expression of NCAM genes (Jones et al., 1992) are consistent with this hypothesis.

In summary, we have documented the involvement of NCAM in limb cell aggregation and in the formation of prechondrogenic condensations. Over- and underex-

Fig. 7. Electroporation of pEC1402 increases the size of precartilaginous condensations and chondrogenic differentiation. Micromass cultures were electroporated with pCH110, encoding β -galactosidase (A-D) or pEC1402, encoding NCAM (E-H) and grown for 4 days. Cultures were stained for the presence of NCAM (B, F), tenascin (C, G), and collagen type II (D, H). The texture of the Millipore filter led to the blurriness of the image. Phase contrast micrographs show that the electroporation of pEC1402 resulted in the formation of much larger precartilaginous aggregates. The electroporated cultures also expressed more NCAM, tenascin, and collagen II than the control cultures. Shown is the same field from phase and fluorescent images of double labelled control (A, B, D) and NCAM electroporated cultures (E, F, G). Figures C and H are taken from parallel control and experimental cultures. Bar: (D) 200 μ m.

pression of NCAM activity can modulate chondrogenic differentiation which appears to be a secondary effect to the aggregate forming behavior of NCAM. Upstream to NCAM, activin, TGF β , retinoic acid, and Hox genes have been shown to be able to regulate the expression of NCAM, either in the limb or nonlimb scenarios (Roubin et al., 1990; Chuong et al., 1992a; Jones et al., 1992; Jiang et al., 1993). Among other molecular events in chondrogenesis, it may be through the modulatory effect of NCAM that these genes shape the morphology of precartilaginous condensations either directly or indirectly, and hence mold the future skeletal patterns. Further experiments will be directed to test the hypothesis that NCAM is a morphoregulatory molecule in limb chondrogenesis.

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LITERATURE CITED

- Ahrens, P.B., Solursh, M., and Reiters, R. (1977) Stage-related capacity for limb chondrogenesis in cell culture. *Dev. Biol.*, *60*:69–82.
- Aulthouse, A.L., and Solursh, M. (1987) The detection of a precartilage, blastema-specific marker. *Dev. Biol.*, *120*:377–384.
- Balling, R., Mutter, G., Gruss, P., and Kessel, M. (1989) Craniofacial abnormalities induced by ectopic expression of the homeobox gene Hox-1.1 in transgenic mice. *Cell*, *58*:337–347.
- Brackenbury, R., Thiery, J.-P., Rutishauser, U., and Edelman, G.M. (1977) Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. *J. Biol. Chem.*, *252*:6835–6840.
- Chiquet, M., and Fambrough, D. (1984) Chicken myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.*, *98*:1926–1936.
- Chisaka, O., and Capecchi, M.R. (1991) Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. *Nature*, *350*:473–479.
- Chuong, C.-M. (1990) Adhesion molecules (NCAM and tenascin) in embryonic development and tissue regeneration. *J. Craniofac. Genet. Dev. Biol.*, *10*:147–161.
- Chuong, C.-M., and Chen, H.M. (1991) Enhanced expression of neural cell adhesion molecules and tenascin (cytotactin) during wound healing. *Am. J. Pathol.*, *138*:427–440.
- Chuong, C.-M., and Edelman, G.M. (1985) Expression of cell adhesion molecules in embryonic induction. I. Morphogenesis of nestling feathers. *J. Cell Biol.*, *101*:1009–1026.
- Chuong, C.-M., Ting, S.A., Widelitz, R.B., and Lee, Y.S. (1992a) Mechanism of skin morphogenesis: II. Retinoic acid gradient modulates axis orientation and phenotypes of skin appendages. *Development*, *115*:839–852.
- Chuong, C.-M., Widelitz, R.B., Jiang, T.-X., Abbott, U., and Lee, Y.S. (1992b) Roles of adhesion molecules NCAM and tenascin in limb chondrogenesis: Analyses with antibody perturbation, transgene expression, talpid mutants and activin stimulation. Proceedings of the 4th International Conference of Limb Development and Regeneration. J.F. Fallon, P.F. Goetinck, R.O. Kelley, and D.L. Stocum, eds. John Wiley and Sons, Inc., New York, (in press).
- Coelho, C.N.D., and Kosher, R.A. (1991) Gradient of gap junctional communication along the anterior-posterior axis of the developing chick limb bud. *Dev. Biol.*, *148*:529–535.
- Crossin, K.L., Chuong, C.-M., and Edelman, G.M. (1985) Expression sequences of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA*, *82*:6942–6946.
- Dickson, G., Peck, D., Moore, S.E., Barton, C.H., and Walsh, F.S. (1990) Enhanced morphogenesis in NCAM-transfected mouse myoblasts. *Nature*, *344*:348–351.
- Ede, D.A., and Flint, O.P. (1975) Cell movement and adhesion in the developing chick wing bud: Studies on cultured mesenchyme cells from normal and *Talpid* mutant embryos. *J. Cell Sci.*, *18*:301–313.
- Edelman, G.M. (1992) Morphoregulation. *Dev. Dynamics*, *193*:2–10.
- Edelman, G.M., Murray, B.A., Mege, R.-M., Cunningham, B.A., and Gallin, W.J. (1987) Cellular expression of liver and neural cell adhesion molecules after transfection with their cDNAs results in specific cell-cell binding. *Proc. Natl. Acad. Sci., USA*, *84*:8502–8506.
- Frenz, D.A., Akiyama, S.K., Paulsen, D.F., and Newman, S.A. (1989a) Latex beads as probes of cell surface-extracellular matrix interactions during chondrogenesis: Evidence for a role for amino-terminal heparin-binding domain of fibronectin. *Dev. Biol.*, *136*:87–96.
- Frenz, D.A., Jaikaria, N.S., and Newman, S.A. (1989b) The mechanism of precartilage mesenchymal condensation: A major role of interaction of the cell surface with the amino-terminal heparin-binding domain of fibronectin. *Dev. Biol.*, *136*:97–103.
- Goulding, M.D., and Gruss, P. (1989) The homeobox in vertebrate development. *Cell Differentiation*, *1*:1088–1093.
- Hamburger, V., and Hamilton, H.L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.*, *88*:49–92.
- Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 626–629.
- Hassell, J.R., and Horigan, E.A. (1982) Chondrogenesis: A model developmental system for measuring teratogenic potential of compounds. *Teratogenesis Carcinog. Mutagen.*, *2*:325–331.
- Hinchliffe, J.R., and Johnson, D.R. (1980) *The Development of the Vertebrate Limb*. Oxford University Press, New York.
- Izpisua-Belmonte, J.-C., Tickle, C., Dolle, P., Wolper, L., and Duboule, D. (1991) Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature*, *350*:585–589.
- Jiang, T.-X., and Chuong, C.-M. (1992) Mechanism of skin morphogenesis. I. Analyses with antibodies to adhesion molecules tenascin, N-CAM, and integrin. *Dev. Biol.*, *150*:82–98.
- Jiang, T.-X., Yi, J.-R., Ying, S.-Y., and Chuong, C.-M. (1993) Activin enhances chondrogenesis of limb bud cells: Stimulation of precartilaginous mesenchymal condensations and expression of NCAM. *Dev. Biol.*, *155*:545–557.
- Jones, F.S., Prediger, E.A., Bittner, D.A., De Robertis, E.M., and Edelman, G.M. (1992) Cell adhesion molecules as targets for Hox genes: Neural cell adhesion molecule promoter activity is modulated by cotransfection with Hox-2.5 and 2.4. *Proc. Natl. Acad. Sci. USA*, *89*:2086–2090.
- Joyce, M.E., Roberts, A.B., Sporn, M.B., and Bolander, M.E. (1990) Transforming growth factor-B and the initiation of chondrogenesis and osteogenesis in the rat femur. *J. Cell Biol.*, *110*:2195–2207.
- Keane, R.W., Mehta, P.P., Rose, B., Honig, L.S., Loewenstein, W.R., and Rutishauser, U. (1988) Neural differentiation, NCAM mediated adhesion, and gap junctional communication in neuroectoderm. A study in vitro. *J. Cell Biol.*, *106*:1307–1319.
- Kessel, M., Balling, R., and Gruss, P. (1990) Variations of cervical vertebrae after expression of a Hox-1.1 transgene in mice. *Cell*, *61*:301–308.
- Knudson, C.B., and Toole, B.P. (1985) Changes in the pericellular matrix during differentiation of limb bud mesoderm. *Dev. Biol.*, *112*:308–318.
- Kosher, R.A., Gay, S.W., Kamanitz, J.R., Kulyk, W.M., Rodgers, B.J., Sai, S., Tanaka, T., and Tanzer, M.L. (1986) Cartilage proteoglycan core protein gene expression during limb cartilage differentiation. *Dev. Biol.*, *118*:112–117.
- Kulyk, W.M., and Kosher, R.A. (1987) Temporal and spatial analysis of hyaluronidase activity during development of the embryonic chick limb bud. *Dev. Biol.*, *120*:535–541.
- Lawrence, M.B., and Springer, T.A. (1991) Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell*, *65*:859–873.
- Lee, Y.S., and Chuong, C.-M. (1992) Adhesion molecules in skeletogenesis: 1. Transient expression of neural cell adhesion molecule

- (NCAM) in osteoblasts during endochondral and intramembranous ossification. *J. Bone Miner. Res.*, *7*:1435-1446.
- Le Mouellic, H.L., Lallemand, Y., and Brulet, P. (1992) Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. *Cell*, *69*:251-264.
- Leonard, C., Fuld, H.M., Frenz, D.A., Downie, S.A., Massague, J., and Newman, S.A. (1991) Role of transforming growth factor-B in chondrogenic pattern formation in the embryonic limb: Stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGF-B and evidence for endogenous TGF-B like activity. *Dev. Biol.*, *145*:99-109.
- Lev, R., and Spicer, S.S. (1964) Specific staining of sulfate groups with Alcian blue at low pH. *J. Histochem. Cytochem.*, *12*:38-41.
- Mackie, E.J., Thesleff, I., and Chiquet-Ehrismann, R. (1987) Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogenesis in vitro. *J. Cell Biol.*, *105*:2569-2579.
- Maier, C.E., Watanabe, M., Singer, M., McQuarrie, I.G., Sunshine, J., and Rutishauser, U. (1986) Expression and function of neural cell adhesion molecules during limb regeneration. *Proc. Natl. Acad. Sci., USA*, *83*:8395-8399.
- McGinnis, W., and Krumlauf, R. (1992) Homeobox genes and axial patterning. *Cell*, *68*:283-302.
- Nah, H.-D., Rodgers, B.J., Kulyk, W.M., Kream, B.E., Kosher, R.A., and Upholt, W.B. (1988) In situ hybridization analysis of the expression of the type II collagen gene in the developing chicken limb bud. *Collagen Relat. Res.*, *8*:277-294.
- Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T., and Taniguchi, S. (1991) Involvement of the Chox-4 chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell*, *64*:1197-1295.
- Paulsen, D.F., Langille, R.M., Dress, V., and Solursh, M. (1988) Selective stimulation of in vitro bud chondrogenesis by retinoic acid. *Differentiation*, *39*:123-130.
- Roubin, R., Deagostini-Bazin, H., Hirsch, M.-R., and Goridis, C. (1990) Modulation of N-CAM expression by transforming growth factor- β , serum, and autocrine factors. *J. Cell Biol.*, *111*:673-683.
- Rutishauser, U., Acheson, A., Hall, A.K., and Sunshine, J. (1988) NCAM as a regulator of cell-cell interactions. *Science*, *240*:53-57.
- Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.*, *5*:3133-3142.
- Serras, F., Fraser, S., and Chuong, C.-M. Pattern of gap junctional communication studied by microinjection of Lucifer Yellow. *Development* (in press).
- Singley, C.T., and Solursh, M. (1981) The spatial distribution of hyaluronic acid and mesenchymal condensation in the embryonic chick wing. *Dev. Biol.*, *84*:102-120.
- Soler, A.P., and Knudsen, K.A. (1991) Colocalization of N-CAM and N-Cadherin in avian skeletal myoblasts. *Dev. Biol.*, *148*:389-392.
- Summerbell, D. (1974) Interaction between the proximo-distal and antero-posterior coordinates of positional value during the specification of positional value during the specification of positional information in the early development of the chick limb bud. *J. Embryol. Exp. Morphol.*, *32*:227-237.
- Tabin, C.J. (1991) Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. *Cell*, *66*:199-217.
- Thorogood, P.V., and Hinchliffe, J.R. (1975) An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. *J. Embryol. Exp. Morphol.*, *33*:581-606.
- Wozney, J.M., Rosen, V., Celeste, A.J., Mitscock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M., and Wang, E.A. (1988) Novel regulators of bone formation: Molecular clones and activities. *Science*, *242*:1528-1534.
- Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991) Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature*, *353*:443-445.
- Zimmermann, B., and Thies, M. (1984) Alterations of lectin binding during chondrogenesis of mouse limb buds. *Histochemistry*, *81*:353-361.