

Adhesion Molecules in Skeletogenesis: I. Transient Expression of Neural Cell Adhesion Molecules (NCAM) in Osteoblasts During Endochondral and Intramembranous Ossification

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ABSTRACT

We report that neural cell adhesion molecules (NCAM) are expressed transiently in developing chicken osteoblasts during osteogenesis using immunostaining on cryostat sections. NCAM is strongly expressed in most osteoblasts along bone trabeculae that coincide with the presence of collagen I and alkaline phosphatase activity. In endochondral ossification, NCAM is highly expressed in osteogenic buds as seen in the epiphysis and diaphysis of tibia and vertebrae. In intramembranous ossification, NCAM is seen in osteogenic condensation of calvaria and in the periosteum of tibial diaphysis. The expression is transient because NCAM is not expressed in mesenchymal cells before osteogenic condensation and NCAM expression is lost in osteocytes in later stages. The staining pattern suggests that NCAM is present on the cell membrane of osteoblasts. Using a specific monoclonal antibody, the osteoblast NCAM is shown to contain polysialic acid, which is enriched in embryonic brain. Northern blot analysis using chicken brain NCAM cDNA as probes showed two major sizes of mRNA at 6.4 and 4.2 kb in calvarial mRNA as opposed to bands at 7.2, 6.4, and 4.2 kb in the brain. An immunoblot showed major proteins at Mr 165 and 110 kd, unlike brain NCAM, which are 180, 140, and 120 kd. That NCAM is involved in bone morphogenesis is consistent with the general hypothesis that NCAM plays pivotal roles in mesenchymal condensation, as shown in the formation of muscle, kidney, skin, and cartilage. The results establish NCAM as a cell surface molecule expressed transiently during osteoblast lineage. The implication that NCAM may mediate osteoblast interaction and regulate skeletal morphogenesis is discussed.

INTRODUCTION

THE SKELETON constitutes the structural framework of the body. The first sign of skeletogenesis is the formation of mesenchymal condensations. Initiation of the condensation defines the position of the skeletal element, and regulated continuous growth and differentiation define the shape and size of the final skeletal element. In intramembranous ossification, mesenchymal cells aggregate to form the osteogenic precursors directly, and in endochondral ossification, mesenchymal cells form cartilage anlage first, which is subsequently replaced by bone. Osteogenic precursor cells can derive locally, such as sclerotome for vertebrae, or from a distance, such as somatopleure for the limb skeleton and neural crest for the skull. No matter

where the mesenchymal cell originates, once it is committed, the chondroosteogenic precursor cell goes through a series of changes in cellular behavior and molecular expression before it finally becomes a mineralizing osteocyte. The progression of this process is heavily regulated by interactions among cells and between cells and their environments. It is through this growth control process that the aggregated osteocytes are molded into a functional skeleton. To understand the mechanism of skeletal development as well as fracture healing, it is essential that we understand the molecular basis of cell interaction during chondrogenesis and osteogenesis.^(1,2)

In osteogenesis, many cellular and molecular events occur in orderly sequence. The cellular processes of ossification have been studied, and three major periods—prolif-

eration, extracellular maturation, and mineralization – are identified.^(3,4) Many molecules functioning as growth factors, extracellular matrix molecules, or enzymes are expressed in specific time and are required during the transformation from a mesenchymal cell to an osteoblast.⁽⁵⁾ Among these, fibronectin, osteonectin, and tenascin mediate cell-substrate adhesion and are involved in cell-matrix interactions. However, the roles of cell-cell adhesion in osteogenesis and the molecules involved in this adhesion have not been studied in detail. The importance of cell adhesion during osteogenesis is shown by the cell condensation process during the development of the periosteum and calvaria, as well as by the tendency of calvarial cells to form osteogenic nodules *in vitro*.⁽⁶⁾

We have been studying the roles of neural cell adhesion molecules (NCAM) in the morphogenesis of mesenchymal tissues.⁽⁷⁾ NCAM are membrane glycoproteins of the immunoglobulin superfamily that were found to be important in cell interactions during neural development. Perturbation of NCAM led to inhibition of neurite fasciculation and retinotectal formation.⁽⁸⁾ NCAM purified from brain contains three major polypeptides at Mr 180, 140, and 120 polypeptides. When NCAM is purified from embryonic tissues, it contains more sialic acid and is less adhesive.⁽⁸⁾ Subsequently, we showed that NCAM was also expressed in a variety of developing mesenchymal tissues. Most interestingly, it is expressed transiently during different mesenchymal condensations of kidney tubule condensations, muscle condensations, dermal condensations, and precartilaginous condensations.^(7,9,10) Functional perturbation experiments using antibodies to NCAM showed inhibition of dermal condensations.⁽¹¹⁾ During skin wound healing in the adult, we also found that NCAM is reexpressed in the granulation tissue but disappears after the wound heals.⁽¹²⁾

In chondrogenesis, we found that NCAM is transiently expressed during the formation of precartilaginous condensations but disappears in mature chondrocytes. Suppression of this NCAM expression by antibodies led to inhibition of the number and size of precartilaginous condensations as well as chondrogenetic differentiation.⁽¹⁰⁾ In contrast, enhancement of NCAM by transgene expression led to an increase in cartilage nodules.⁽¹⁰⁾ Thus, NCAM is a key molecule in the molecular cascade of chondrogenesis. We therefore ask whether NCAM is also expressed during a specific stage of osteogenesis. If so, what is its specific distribution pattern compared to other osteogenic markers? Is there any difference in its mode of expression in endochondral and intramembranous ossification? Are the biochemical forms of NCAM different from those of brain NCAM? What are the potential roles of this adhesion molecule in skeletogenesis, and how would it be related to other molecules in the molecular cascade of osteogenesis?

MATERIALS AND METHODS

Materials

White Leghorn chicken eggs were obtained from K and R Farm (Westminster, CA), and chicken embryos were staged according to Hamburger and Hamilton.⁽¹³⁾ Rabbit

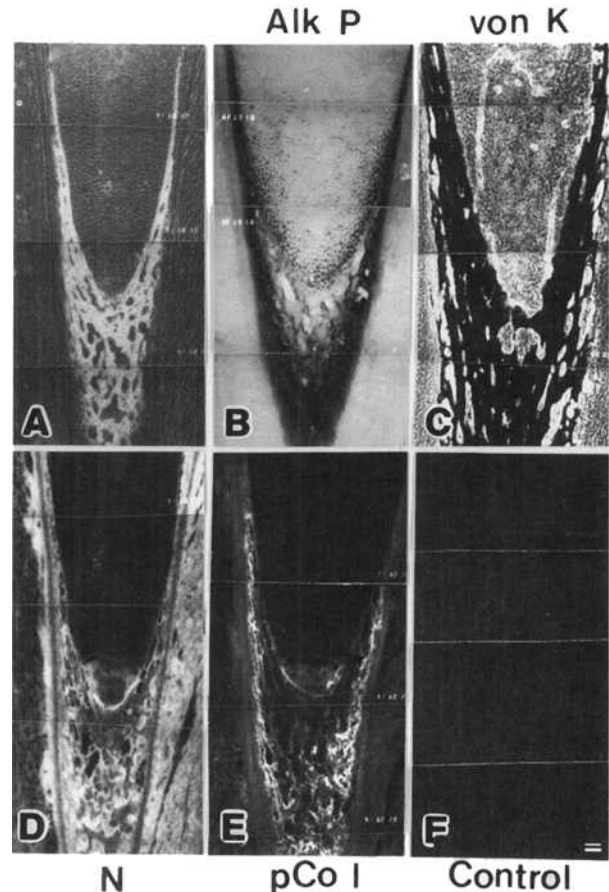


FIG. 1. Expression of NCAM during osteogenesis of long bone. Longitudinal frozen sections of the diaphysis from the tibia of stage 40 chicken embryo. (A) Phase contrast; (B) histochemical staining of alkaline phosphatase (Alk P) activity; (C) von Kossa staining (von K) to show mineralized regions; (D) immunofluorescent staining with rabbit antichick NCAM (N); (E) immunofluorescent staining with mouse antiprocollagen type I (pCoI); (F) control nonimmune staining. The low margin of each panel is about the midpoint level of the tibial shaft. The upper tibia is still cartilaginous, and the lower midtibia has been replaced by bone trabeculae. Note the corresponding staining regions of NCAM, procollagen type I, and alkaline phosphatase. Bar, 100 μ m.

antichick NCAM and monoclonal antibody 15G8 against polysialic epitope of chicken NCAM were prepared as described.^(14,15) Rabbit antichick collagen I was purchased from Chemicon (Temecula, CA). Monoclonal antibodies against sheep procollagen type I (SP1.D8), which cross-react with chicken,⁽¹⁶⁾ chicken tenascin (M1),⁽¹⁷⁾ and chicken fibronectin (B3/D6)⁽¹⁸⁾ were kindly provided by the Developmental Studies Hybridoma Bank (Baltimore, MD). The vector containing NCAM (pEC1402)^(19,20) was kindly provided by Dr. Murray (University of California-Irvine).

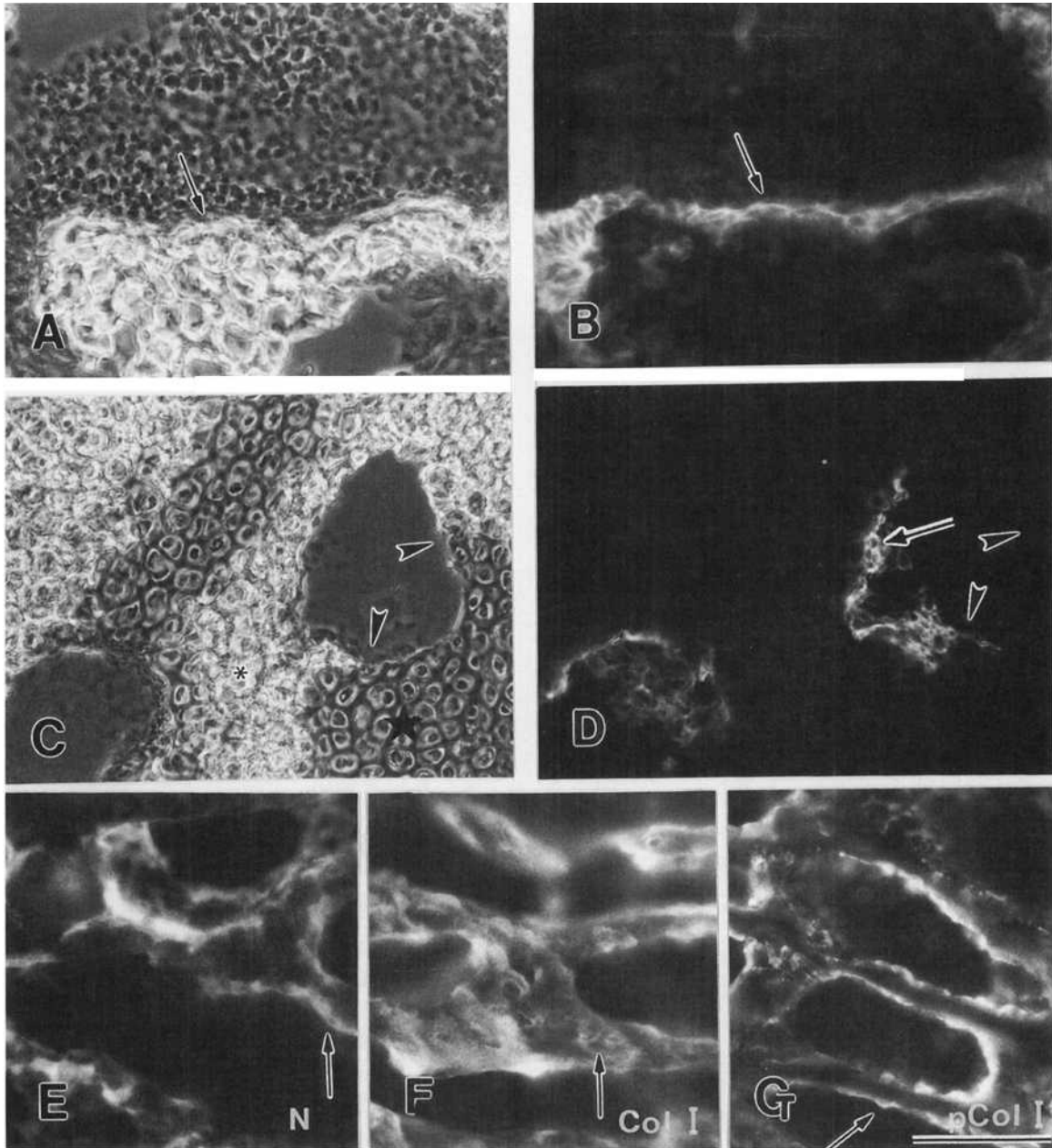


FIG. 2. Expression of NCAM in osteoblasts: (A-D) stage 36 chicken vertebrae, endochondral ossification regions showing cartilage (star), calcified chondrocytes (asterisk), and osteoblasts (arrows); (E-G) stage 40 chicken tibia showing bone trabeculae and osteoblasts (arrows). (A and C) Phase contrast; (B, D, and E) anti NCAM immunofluorescent staining (N); (F) anticollagen I (ColI); (G) antiprocollagen I (pColI). Note that osteoblasts were positive for NCAM (B, D, and E), collagen I (F), and procollagen I (G). These NCAM-positive cells were absent on the surface of cartilage (C and D, flanked by arrowheads). In E-G, the unevenness of the bone trabeculae made it difficult to have the whole picture in the same focal plane. Bar, 100 μ m.

Immunofluorescence

Dissected tissues from the indicated embryonic stages were fixed in 2.5% paraformaldehyde in phosphate-buffered saline, followed by 20% sucrose for another 2 h. The tissues were then embedded in Tissue-Tek (Miles, Elkhart,

IN), chilled by dry ice, and sectioned at 10 μ m with a cryostat. Sections were preincubated in 5% normal goat serum for 1 h and then changed to primary antibodies overnight. After washing, sections were incubated in fluorescein-conjugated goat antirabbit (1:50) or biotinylated horse anti-

mouse antisera followed by Texas-red avidin D (Vector, Burlingame, CA). Specimens were mounted in 90% glycerol containing *p*-phenylenediamine dihydrochloride to prevent the decay of fluorescence.⁽²¹⁾

Histochemistry

Endogenous alkaline phosphatase activity was localized using bromochloroindolyl phosphate and nitroblue tetrazolium as substrates (Vector; Burlingame, CA). Positive staining results in a purple color. The von Kossa staining was performed by immersing slides in silver nitrate and sodium thiosulfate sequentially as described by Kiernan.⁽²²⁾ Neuraminidase treatment of sections was carried out by treating the 2.5% paraformaldehyde sections with neuraminidase (*Vibrio cholerae*; Sigma, St. Louis, MO) at 37°C for 24 h.⁽¹⁵⁾

Immunoprecipitation, immunoblot analysis, and neuraminidase treatment

Dissected long bone and calvaria (300 mg) from stage 39/40 chicken embryos were homogenized in 5 ml ice-cold

extraction buffer (phosphate-buffered saline, 0.5% Nonidet P40, and 1 mM EDTA) containing Trasylol (200 kIU/ml) and phenylmethylsulfonyl fluoride (PMSF, 1 mM) as described.⁽²³⁾ After vortexing and standing in ice for 5 minutes, the samples were centrifuged in a microfuge for 10 minutes. Rabbit antichickens NCAM (1 mg/ml) was added to the supernatant of bone extract (200 μ l) in 1:200 dilution. After overnight incubation at 4°C, 30 μ l protein A bead (Sigma, St. Louis, MO) as 10% vol/vol in extraction buffer was added and incubated at 4°C for 1 h with rocking. Subsequently, the beads were washed four times in extraction buffer and treated with neuraminidase (1 unit) or buffer only at 37°C overnight. After boiling beads in sample buffer proteins were separated on an 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted to nitrocellulose filter according to standard procedures.⁽²³⁾ We used a biotinylated secondary antibody followed by streptavidin-conjugated alkaline phosphatase. The color was developed using bromochloroindolyl phosphate and nitroblue tetrazolium as substrates.

Northern blot

Poly(A)⁺ RNA was purified from dissected chicken calvariae using an acid guanidinium thiocyanate-phenol-chlo-

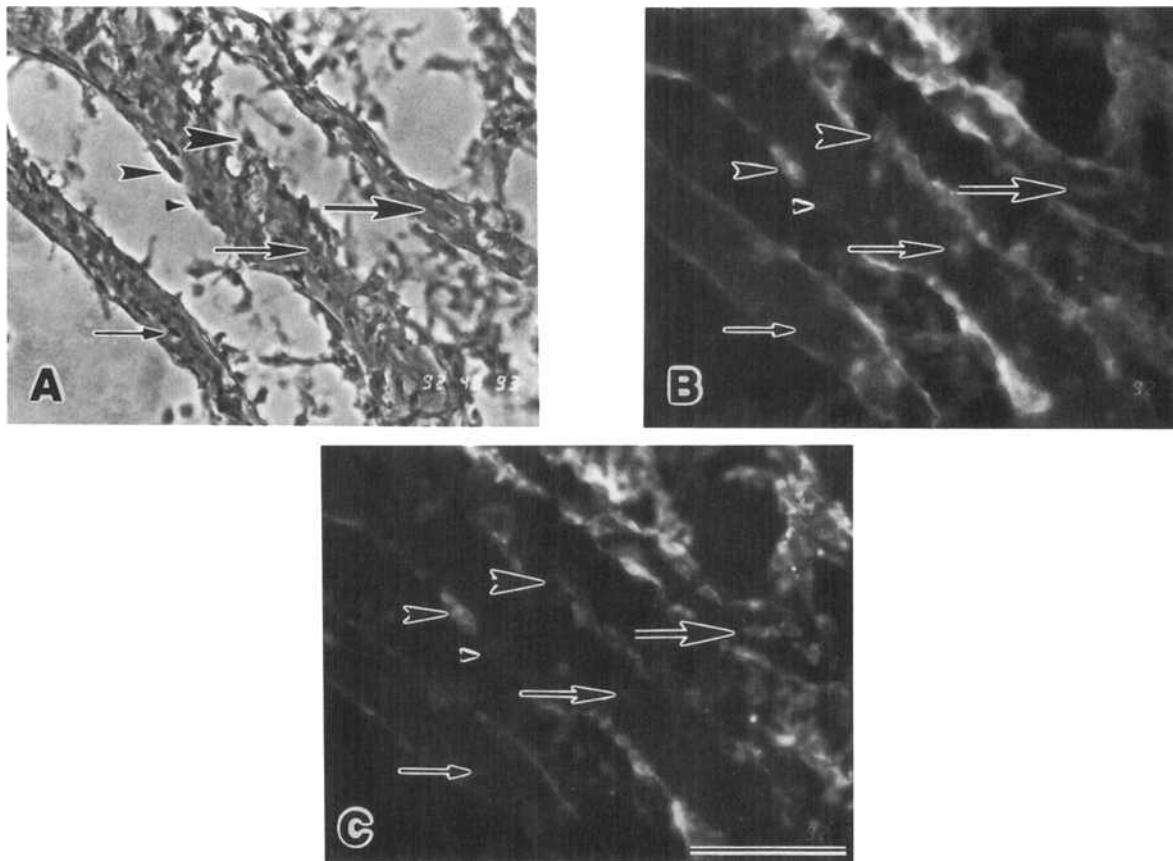


FIG. 3. Osteoblasts double stained with antibodies to procollagen I and NCAM. Longitudinal section of ossified tibia from stage 40 chicken embryo was double stained with anti-NCAM (B) and antiprocollagen I (C). The same section was later subjected to hematoxylin and eosin staining (A). Osteoblast staining: small arrowhead, NCAM⁻ procollagen⁻; medium arrowhead, NCAM⁺ procollagen⁻; large arrowhead, NCAM⁺ procollagen⁻. Osteocyte staining: small arrow, NCAM⁻ procollagen⁻; medium arrow, NCAM⁺ procollagen⁻; large arrow, NCAM⁺ procollagen⁺. Size bar, 100 μ m.

roform method⁽²⁴⁾ and oligo(dT) column (Boehringer Mannheim, Mannheim, Germany). Poly(A)⁺ RNA (3 μ g) was separated on a 1% agarose formaldehyde gel and blotted onto a nitrocellulose membrane.⁽²⁵⁾ The probe used was an BamHI fragment (about 3.7 kb in length) from the NCAM cDNA plasmid pEC1402.^(19,20) Probes were labeled with [α -³²P]dCTP using random priming labeling (Stratagene, La Jolla, CA).

RESULTS

We examined the expression of NCAM during ossification of the axial skeleton (vertebrae), appendicular skeleton (tibia), and calvaria. During chondrogenesis, NCAM is expressed transiently in precartilaginous condensations. NCAM then disappears in chondrocytes but remains in the perichondrium.^(7,10) At the onset of ossification, NCAM is reexpressed in the ossification centers. Using the developing chicken tibia (stage 40) as an example, NCAM was present in endochondral bone formation zone, which is also positive for procollagen I, collagen I, alkaline phosphatase, and von Kossa staining (Fig. 1). NCAM can also be seen in the perichondrium and muscle, but these regions were procollagen I negative. We then examined the expression of NCAM in different ossification centers.

Endochondral bone formation

Higher magnification of the zone of endochondral bone formation and ossified bone trabeculae showed that NCAM was positive on osteoblasts (Fig. 2A-E, arrows). The staining pattern of NCAM on osteoblasts implied cell membrane localization (Fig. 2B, D, and E). These cells were also positive for both collagen I and procollagen I (Fig. 2F and G). Alkaline phosphatase activity was present inside the bone trabeculae (not shown).

The osteoblasts on the bone trabeculae were further studied using double staining of NCAM and procollagen I (Fig. 3). Most cells on the trabecular surface were positive for both NCAM and procollagen I and were considered osteoblasts (Fig. 3, medium arrowhead). Few cells on the trabecular surface with a similar appearance were NCAM positive but procollagen I negative (large arrowhead), or negative in both NCAM and procollagen I (small arrowhead). These cells were small in percentages and may represent different stages of early osteoblast development. Osteocytes within bone trabeculae were mostly NCAM negative, although some cells with faint NCAM staining can be seen (Fig. 3, arrows).

In the long bone as well as in vertebrae, the epiphyseal plates continue to grow new cartilage while ossification centers form in the diaphysis and epiphysis regions by

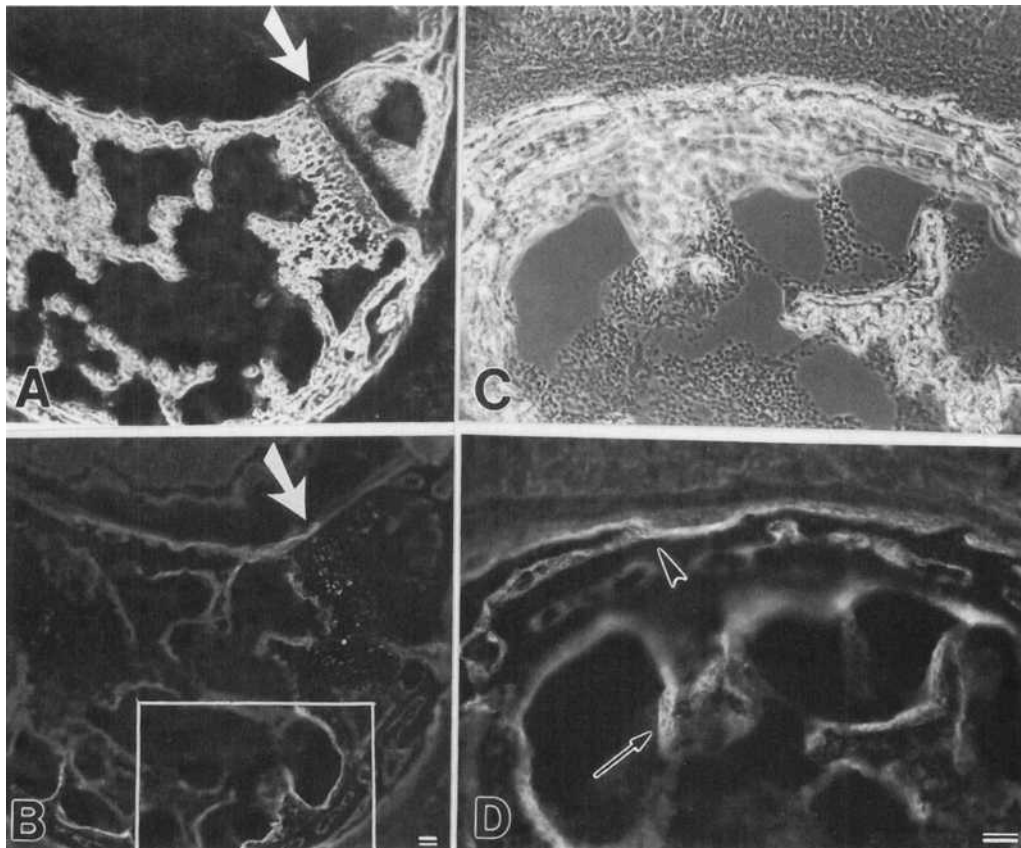


FIG. 4. Ossification centers in the vertebrae. Cross sections of vertebrae from stage 36 chicken embryo. (A and B) Low-power views of a vertebra with an epiphyseal plate (thick arrow). (A) Dark-field illumination adjusted to brighten bone trabeculae; (B) NCAM staining; (D) Enlargement from rectangles in B (rotated); (C) Phase contrast; (D) NCAM staining. Osteogenic sprouts can be seen to arise from the periosteum region (arrowhead in D), and osteoblasts lining bone trabeculae were NCAM positive (arrow in D). (A and B) Bar is 100 μ m; (C and D) bar is 100 μ m.

sprouting osteogenic buds from the periosteum layer. These osteogenic buds were composed of osteogenic tissue and blood capillaries. They invaded into the cartilage zone and eroded away chondrocytes. The preosteoblasts and osteoblasts within the osteogenic sprouts were highly positive for NCAM (Fig. 4D, arrow).

Intramembranous bone formation

During development, on the periphery of the cartilage, mesenchymal cells aggregate to form a layer of dense connective tissue that later becomes the perichondrium and remains NCAM positive (Fig. 5A). As the cartilage primordium is replaced by the bone, the perichondrium is replaced by the periosteum. Progressing from the outside toward the inside, the periosteum is composed of a superficial layer of flattened fibroblasts that are NCAM positive, a middle layer of collagenous fibrils that are NCAM negative, and an inner layer of preosteoblasts and an innermost layer of osteoblasts, which are both NCAM positive (Fig. 5A-C). As the inner osteoblasts became osteocytes, new osteoblasts were generated from the outer periosteum, and thus the shaft of the long bone grew in thickness continuously through this appositional intramembranous bone formation.

To compare different types of ossification and different germ layer lineages of osteoblasts, we further examined NCAM in the intramembranous ossification of calvariae. The calvarial cells are derived from cranial ectomesenchymal cells, which are primarily of neural crest origin. In the earlier stages, a layer of aggregated mesenchymal cells above the brain gradually formed that was positive for NCAM and then became positive for both NCAM and tenascin (Fig. 6A-C). This osteogenic condensation layer gradually thickened and differentiated into skull. Within the skull, NCAM was positive in the periosteum and osteoblasts (Fig. 6D and E). Some cells coexpressed NCAM and tenascin, but NCAM was expressed higher in osteoblasts than in the periosteum and tenascin was expressed higher in the periosteum than in osteoblasts (Fig. 6F). Later, in osteocytes, NCAM again became negative (not shown). Thus, despite the neural crest origin of the skull, the expression sequence of adhesion molecules during osteogenesis remains the same. We also examined various ossification centers of mouse and rat with antibodies to mouse NCAM and found that NCAM was also present in the osteoblasts of these rodents (not shown).

Biochemical characterization of osteoblast NCAM

To initially characterize osteoblast NCAM, we tried to identify its protein species, mRNA species, and polysialic acid.^(14,23,26) Developing bones from stage 39 and 40 chicken embryos were pooled and homogenized. The extracts were resolved by polyacrylamide gel electrophoresis. Two major bands at Mr 165 and 110 kD and some minor bands between them were identified (Fig. 7). This is within the size of NCAM identified in the brain, skin, and muscle. A diffuse band spreading between Mr 140,000 and 200,000 can also be seen. When treated with neuramini-

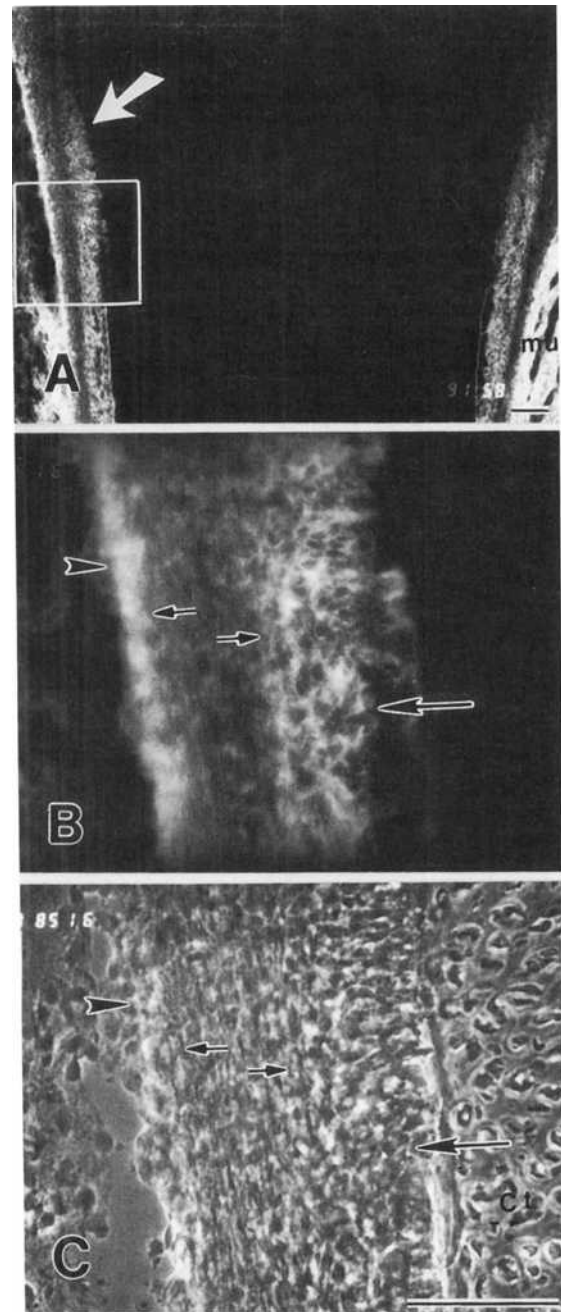


FIG. 5. Periosteal bone formation. Longitudinal sections of stage 40 embryonic chicken tibia. (A and B) NCAM staining; (C) phase contrast. B and C are from the rectangle of A. This figure showed the transition from perichondrium (A, thick arrow) into periosteum (lower part of A). In the periosteum (B and C), there was a superficial NCAM-positive flat fibroblast layer (flanked by arrowhead and small arrow), a NCAM-negative fibril layer (flanked by two small arrows), and an inner NCAM-positive osteogenic layer (flanked by small and large arrows). Bar is 100 μ m in A; mu muscle. Bar is 100 μ m in B and C.

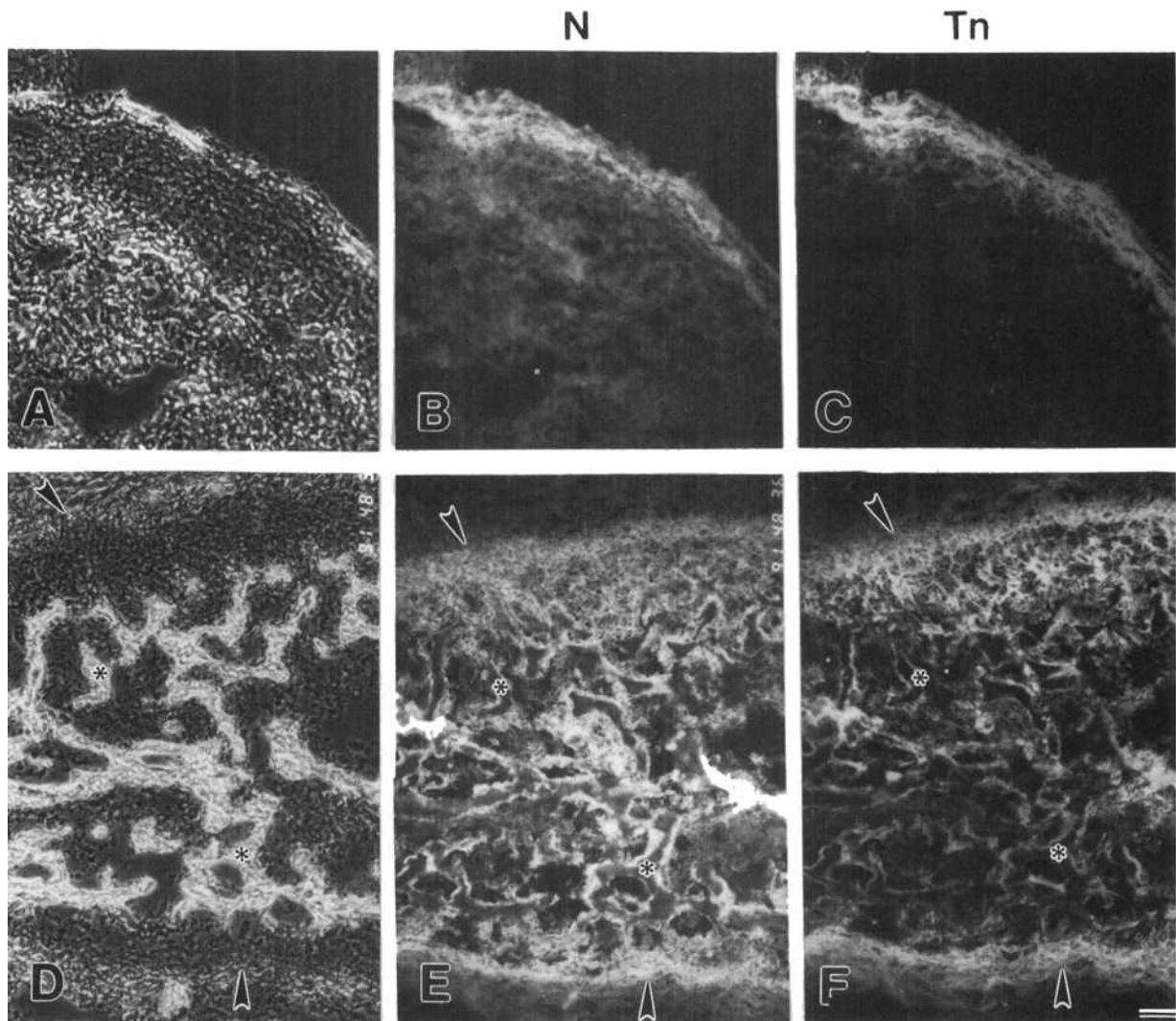


FIG. 6. Calvaria formation. Longitudinal sections of the skull from stage 43 chicken embryo. (A–C) Calvaria over anterior head where skull formation was in its early stage; (D–F) calvaria over posterior head where skull formation was more mature; (A and D) phase contrast; (B and E) anti-NCAM; (C and F) same section double stained with antitenascin. Some condensed osteogenic mesenchymal cells were positive for NCAM and negative for tenascin (B and C, right part of the micrograph). Some were positive for both NCAM and tenascin (B and C, left part of the micrograph). In the differentiating calvariae, both NCAM and tenascin were positive in the periosteum (arrowhead) and osteoblasts over the trabecular region (asterisk), although tenascin was weaker in the trabecular region. A similar tenascin staining pattern was previously observed in calvariae.^(51,52) Bar is 100 μ m.

dase, the diffuse band disappears and clear bands emerged. A similar diffuse band was seen in NCAM purified from embryonic brain. The diffuse band appearance is due to the heterogeneous sizes of polysialic acid.^(14,15)

To further test whether osteoblast NCAM contains polysialic acid, we treated fixed cryosections with *Vibrio cholerae* neuraminidase. Before treatment, osteoblasts were positive when reacted with rabbit polyclonal antibodies against NCAM or with mouse monoclonal antibodies specific to the polysialic acid portion of NCAM.⁽¹⁵⁾ After treatment with neuraminidase, the sections remained posi-

tive with polyclonal antibodies, although the intensity somewhat diminished. Monoclonal antibodies to polysialic acid became totally negative. Mock treatment with the same buffer and time of incubation did not abolish antibody staining (Fig. 8).

Messenger RNA from embryonic chicken calvariae were purified and separated on agarose gels. For the brain, three bands at 7.2, 6.4, and 4.2 kb can be identified.⁽²⁷⁾ For the calvariae, two major bands at 6.4 and 4.2 kb were identified, which is consistent with the smaller molecular weight of bone NCAM.

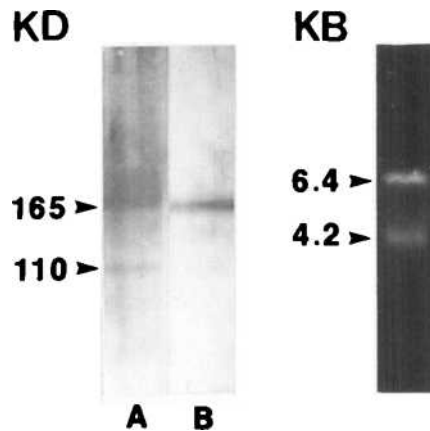


FIG. 7. Immunoblot and Northern blot analysis of NCAM from bone extracts. Bone from all long bones and calvariae from stage 39/40 chicken embryo were dissected, extracted, and immunoprecipitated according to the procedures described in Materials and Methods. Left, immunoprecipitated proteins were resolved by SDS and 7.5% polyacrylamide gel electrophoresis, blotted to nitrocellulose paper, and reacted with antibodies to NCAM followed by alkaline phosphatase-conjugated secondary antibodies. Two major bands at Mr 165 and 110 kD and minor bands between them can be identified (lane A). If NCAM on protein A beads were treated with neuraminidase, the diffuse zone between Mr 140 and 200 kD disappeared and a clear major band at Mr 165 kD with some faint smaller bands can be seen (lane B). Control experiments incubated in enzyme buffer only still showed the characteristic diffuse pattern despite some degree of protein degradation. Right, poly(A)RNA was purified using a poly(dT) column and resolved by 1% agarose-denatured gel electrophoresis, transferred to nitrocellulose, and probed with the NCAM cDNA using an insert isolated as a BamHI fragment from pEC1402. Two major species at 6.4 and 4.2 kb were identified. The 7.2 kb mRNA species corresponding to the Mr 180 kD polypeptide found in the brain was not seen.

DISCUSSION

NCAM was originally found in retina cells and was shown to mediate neurite connection patterns during formation of the nervous system.⁽⁸⁾ Recently we found that NCAM is also a mesenchymal condensation molecule involved in various types of mesenchymal morphogenesis involving skin, kidney, and, in particular, cartilage.^(7,10,11) In this study, we examined the expression pattern of NCAM during osteogenesis of embryonic chicken. We found that NCAM is indeed transiently expressed in osteoblasts during osteogenesis. We identified this specific stage with morphologic and molecular markers. We also compared NCAM expression in different modes of ossification and did preliminary biochemical characterization of osteoblast NCAM. The potential roles of NCAM in osteogenesis and its possible regulation by growth factors and homeobox genes are discussed.

NCAM is transiently expressed during osteogenesis

To compare the expression of NCAM with other osteoblastic phenotype markers, we stained chicken embryo tibia, vertebra, and calvaria sections with antibodies to NCAM, procollagen type I, collagen type I, and tenascin and determined alkaline phosphatase activity and von Kossa staining in adjacent sections. In the long bone, NCAM is originally homogeneously expressed at low levels in limb buds. NCAM then becomes enriched in precartilaginous condensations but disappears when the condensed mesenchymal cells become chondroblasts and chondrocytes.^(7,10) NCAM remains positive in the perichondrium. Around stage 40, endochondral ossification is observed in the diaphyseal region of the tibia and the bone trabeculae are highly NCAM positive (Fig. 1). Further examination showed that these cells are osteoblasts. Collagen type I and procollagen I were used as markers to confirm that the majority of these cells are osteoblasts (Figs. 1 through 3). However, some of the NCAM-positive cells on the trabecular surface are procollagen I negative. A small percentage of these cells were even negative in both NCAM and procollagen I. This heterogeneity in staining may represent different stages of early osteoblast development, although further investigation with more molecular markers are needed. When osteoblasts mature into osteocytes, NCAM becomes very weak in a few osteocytes and negative in most osteocytes (Fig. 3). This observation was confirmed in our osteoblast culture study (manuscript in preparation). These results reflect the transient expression of NCAM during osteogenesis, which implies the usefulness of NCAM as a marker for osteogenesis.⁽⁴⁾

Intramembranous ossification also occurred in the diaphysis. The innermost layer of the periosteum generates osteoblasts that contribute to thickening of the bone cortex. This layer of osteoblasts is also positive for NCAM (Fig. 5). In the formation of vertebrae, the sclerotome is positive for NCAM and forms precartilaginous condensations (our unpublished data). At stage 36, the major portion of the cartilaginous vertebral body is replaced by numerous osteogenic buds, except in the epiphyseal region, where cartilage continues to grow to form vertebral processes. The osteogenic buds contain NCAM-positive osteoblasts, and the budding of these cells from the periosteum region can be seen (Fig. 4). Before the formation of calvariae, there is a loose layer of mesenchymal cells above the brain, and these mesenchymal cells are NCAM negative. These mesenchymal cells gradually condense to form osteogenic condensations that are highly NCAM positive. This calvarial condensation then thickens and generates the bone trabeculae and periosteum, both NCAM positive (Fig. 6). Again, NCAM disappears from mature osteocytes. A parallel sequence of expression of NCAM in osteoblasts can also be seen in cultured calvarial cells stimulated by ascorbic acid and β -glycerophosphate. Using double staining, NCAM was expressed after fibronectin and tenascin but at about the same time as collagen type I (manuscript in preparation).

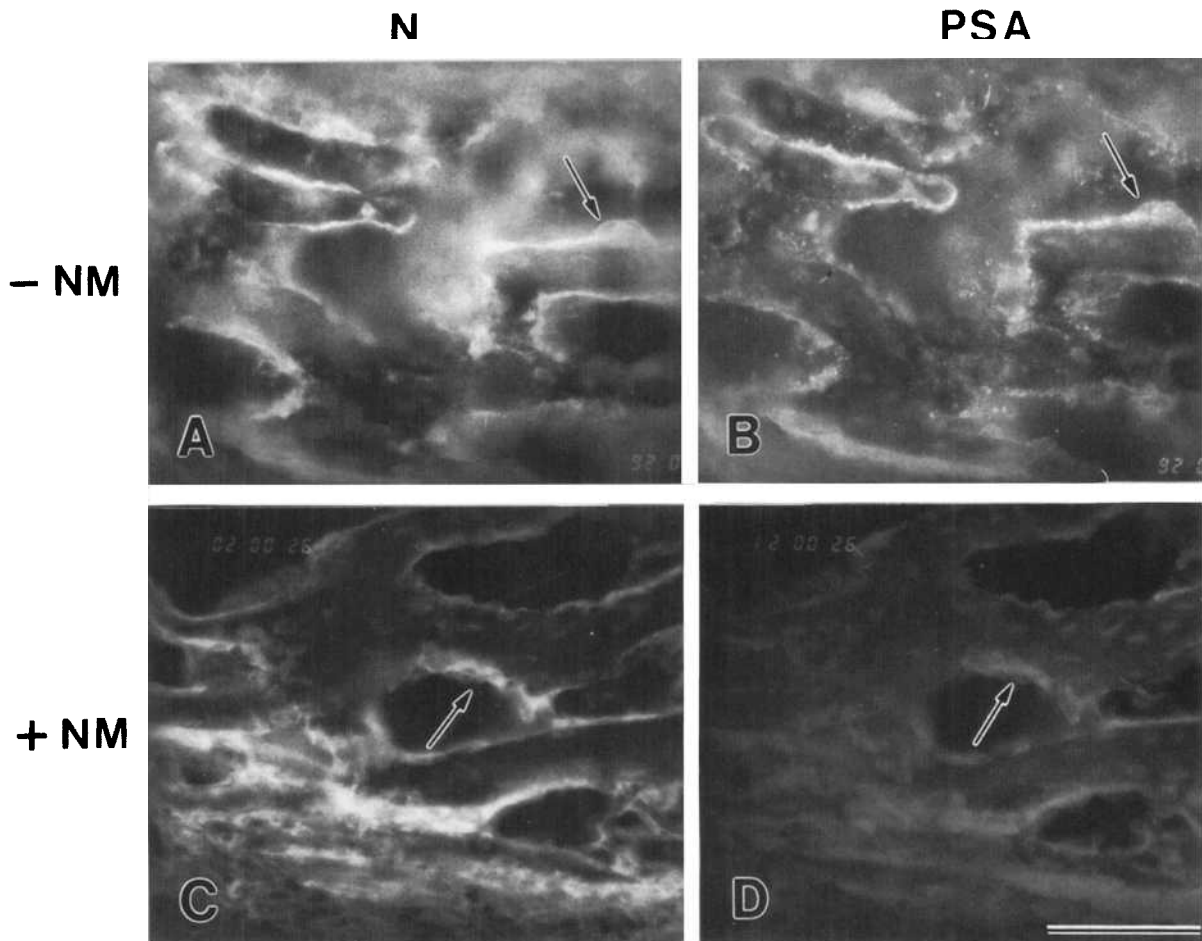


FIG. 8. Osteoblast NCAM contains an epitope that is removable by neuraminidase. Stage 40 embryonic chicken tibia sections were treated with neuraminidase (C and D) or only neuraminidase buffer (A and B) and double stained with rabbit polyclonal antibody to NCAM (A and C) and monoclonal antibody 15G8 (B and D) specific to the polysialic acid residue (PSA).⁽¹⁵⁾ The control showed very similar distributions of the two antibodies. The plasma membrane staining patterns of some osteoblasts are indicated by arrows. Note that 15G8 binding disappears after neuraminidase treatment. The unevenness of bone trabeculae caused some portions to be out of focus. NM, neuraminidase. Bar is 100 μ m.

Expression of NCAM in osteoblasts is independent of different germ layer origins and ossification modes

Most of the osteoblasts in skull are derived from neural crest cells, which are ectodermal in origin. Osteoblasts in vertebrae derive from somites, and osteoblasts in limb skeleton derive from somatopleure. Despite these different origins, NCAM is expressed in osteoblasts in skull, vertebrae, and limbs. The expression of NCAM is also consistent whether bone formation results from direct intramembranous bone formation or indirect endochondral (or intracartilaginous) bone formation. Therefore NCAM is expressed in osteoblasts from different embryonic germ layer origins and ossification modes. This suggests that NCAM plays fundamental roles in cell interactions during osteoblast differentiation.

Osteoblast NCAM has specific biochemical forms

Although there is only one NCAM gene, there are different NCAM mRNAs in different tissues generated by differential splicing.⁽²⁷⁾ For the avian brain, RNAs are found at 7.2, 6.4, and 4.2 kb, representing Mr 180, 140, and 120 kD polypeptides.⁽²⁸⁾ The Mr 180 kD appears to be neural specific. NCAM isolated from mesenchymal tissues are in the range 110–160 kD.^(9,29) For the bone, we observed two major mRNA species of 6.4 and 4.2 kb and two major protein bands at Mr 165 and 110 kD. Another mesenchymal NCAM isolated from skin is a dominant Mr 140 kD band.⁽⁹⁾ Using human muscle NCAM for further comparison, there are messages at 6.7, 5.2, and 4.3 kb and polypeptides at Mr 155, 145, and 125 kD. In addition, a stretch of 37 amino acids forms a muscle-specific domain and is only found in muscle NCAM.⁽²⁹⁾ These isoforms

may mediate different functions. For example, when myoblast fusion occurs, the 5.2 and 4.3 kb isoforms increase but the 6.7 kb isoform decreases.⁽²⁹⁾ For fibronectin, different integrin specificities have been reported for different isoforms.⁽³⁰⁾ The structural difference and functional significance of osteoblast NCAM isoforms await further investigation. Another biochemical variation of NCAM is carbohydrate modification,^(26,31) which also shows tissue specificity.⁽²³⁾ The most interesting is the polysialic acid enriched in embryonic brain. NCAM molecules enriched with polysialic acid bind each other with lower affinity than those without polysialic acid,⁽³¹⁾ and it was hypothesized that this is one of the mechanisms that modulate cell adhesion in morphogenesis.⁽⁸⁾ Here we used a polysialic acid-specific monoclonal antibody⁽¹⁵⁾ to show that NCAM in osteoblasts contains polysialic acid residues. These carbohydrate moieties on osteoblast NCAM, similar to those in neural morphogenesis,^(32,33) may be used to regulate bone morphogenesis by modulating the hierarchy of cell-cell and cell-matrix adhesion.

Potential roles of NCAM in the molecular cascade of skeletogenesis

To find the function of NCAM in osteogenesis is the next major question. In neural development, NCAM is involved in neurite fasciculation,⁽³²⁾ retinotectal projection,⁽³⁴⁾ and neural muscular interaction.⁽³⁵⁾ In mesenchymal development, NCAM is involved in kidney tubule condensation,⁽⁷⁾ myoblast fusion,⁽³⁶⁾ skin appendage dermal condensation,⁽¹¹⁾ and precartilaginous condensation.⁽³⁷⁾ In view of these known functions of NCAM, we suspect that the function of the transient expression of NCAM in osteoblasts is to mediate key cell interactions required at a specific stage of osteogenesis. For example, NCAM is not detected until the late osteoblast proliferative phase (manuscript in preparation; phases are defined as described in Ref. 6). Recently, NCAM was shown to mediate contact inhibition in fibroblastic cell lines.⁽³⁸⁾ NCAM may mediate the signal that switch osteoblasts from proliferation to differentiation phase. Binding through NCAM has also been shown to be prerequisite for gap junction formation in neural plate formation.⁽³⁹⁾ Gap junctions are present in osteoblasts *in vivo*⁽⁴⁰⁾ and *in vitro*⁽⁴¹⁾ and may transmit the molecular messengers critical for bone development. Blocking NCAM activity in osteoblasts may prevent bone formation through inhibiting gap junction formation. The expression pattern of NCAM is consistent with these hypotheses, and we are currently exploring these exciting possibilities with NCAM antibodies and cDNA probes.

Peptide growth factors of the transforming growth factor β (TGF- β) superfamily and homeobox-containing transcription factors have recently been characterized and appear to have a profound effect on the differentiation and pattern determination of skeletal formation. Because NCAM is expressed transiently during osteogenesis, 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 down-

stream effects of these molecules. TGF- β and several bone morphogenetic proteins can induce osteogenesis,^(42,43) and their gene structures show that they belong to the TGF- β superfamily. Studies on 3T3 fibroblasts showed that TGF- β can increase the transcription of NCAM.⁽⁴⁴⁾ Thus NCAM expression in osteoblasts may mediate the osteogenic effect of TGF- β members. Recently, we also found that activin can increase NCAM expression and enhance chondrogenesis.⁽⁴⁵⁾ Another connection comes from the recent progress in homeobox genes, which has led to the hypothesis that certain combinations of homeobox genes (Hox code) determine the pattern of limb and axial skeleton.^(46,47) When Hox codes are disrupted by retinoic acid, the morphology of lumbar vertebrae are altered to become thoracic vertebrae, which grow ribs.⁽⁴⁸⁾ Because a Hox binding region is identified in the 5' region of NCAM genes⁽⁴⁹⁾ and transfection of Hox genes in cell lines can alter the expression of NCAM,⁽⁵⁰⁾ it is compelling to speculate that NCAM may be the downstream molecule mediating the phenotype-determining effect of Hox genes during skeletal morphogenesis.

In summary, we reported here the novel finding that NCAM is expressed in a specific temporal and spatial pattern during osteogenesis. This is consistent with the hypothesis that NCAM is a common regulator of mesenchymal morphogenesis. It is transiently expressed during early morphogenesis of skin, kidney, muscle, cartilage, and now, bone. Among the molecules expressed during the osteoblast lineage, NCAM should now be considered an additional key player. The potential roles of NCAM in mediating osteoblast adhesion interaction and regulating skeletal pattern formation should be critical issues in future research.

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