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Original Article

N-CAM is not required for initiation of secondary chondrogenesis: the role of N-CAM in skeletal condensation and differentiation

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ABSTRACT Condensation precedes chondrogenic differentiation during development of primary cartilage. While neural cell adhesion molecule (N-CAM) enhances condensation, it is unclear whether N-CAM is also required for initiation of chondrogenic differentiation. In this study, the role of N-CAM in secondary chondrogenesis from periosteal cells of the quadratojugal (QJ) from embryonic chicks was studied using several *in vitro* approaches. The QJ is a membrane bone and so is not preceded by cartilage formation during development. However, QJ periosteal cells can differentiate into chondrocytes to form secondary cartilage *in vivo*. When QJ periosteal cells were enzymatically released and plated in low density monolayer, clonal or agarose cultures, chondrogenesis was initiated in the absence of N-CAM expression. Furthermore, overexpression of the N-CAM gene in periosteal cells in monolayer culture significantly reduced the number of chondrocyte colonies, suggesting that N-CAM inhibits secondary chondrogenesis. In contrast, and consistent with expression *in vivo*, N-CAM is expressed during osteogenesis from QJ periosteal cells and mandibular mesenchyme *in vitro*. These results are discussed in relation to the role of N-CAM in osteogenesis and in primary and secondary condensation.

KEY WORDS: *cell adhesion molecules, N-CAM, chondrogenesis, cartilage, osteogenesis, bone, morphogenesis*

Introduction

During embryogenesis, cartilage develops from mesenchyme derived from both mesoderm and neural crest. Mesodermal mesenchyme gives rise to cartilages in the trunk and a few cartilages in the craniofacial regions. Most craniofacial cartilages are neural crest derivatives (Le Lièvre, 1978; Noden, 1978, 1988; Couly *et al.*, 1993; Le Douarin *et al.*, 1993, Hall, 1999). Regardless of their diverse embryonic origins or locations, mesenchymal cells first undergo precartilaginous condensation before chondroblasts emerge from the condensed mesenchymal cells (Thorogood and Hinchliffe, 1975; Ede, 1983; Hall and Miyake, 1992, 1995). Chondroblasts are identified by expression of cartilage phenotypic genes, such as type II collagen and cartilage-specific proteoglycans. Most cartilages are replaced by bone through endochondral ossification; only a few remain as permanent cartilages in adults. Hence cartilage is often the template for bone formation and as such plays an important role in morphogenesis, growth, remodeling, and fracture healing of bone (Erlebacher *et al.*, 1995).

Mesenchymal condensation followed by chondrogenesis is the common sequence for all primary cartilages. However, in membrane bones of the craniofacial skeleton, cartilages arise from preexisting periostea at articular surfaces following initial osteogenesis. Two examples are mammalian mandibular condylar cartilage (Livne and Silbermann, 1990) and cartilage on the avian quadratojugal (QJ) (Murray, 1963; Hall, 1979; Fang and Hall, 1995). Such cartilages have been termed secondary cartilage to distinguish them from primary cartilage. During secondary chondrogenesis, chondroblasts differentiate from periostea of membrane bone rather than from condensed mesenchyme (Beresford, 1981; Fang and Hall, 1997). Secondary cartilages function as growth centers and as articular cartilages for the membrane bones of the craniofacial skeleton. Initiation and/or maintenance of secondary cartilage depend on biochemical stimulation generated by embryonic movement (Hall, 1979; Livne and Silbermann, 1990; Fang and Hall, 1997).

Abbreviations used in this paper: ABC, avidin-biotin-peroxidase complex; DAB. 3,3'-diaminobenzidine; ECM, extracellular matrix; FBS, foetal bovine saline; HBQ, Hall-Brunt quadruple stain; N-cam, neural cell adhesion molecule; PBS, phosphate buffered saline; PLP, periodate-lysine-paraformaldehyde; QJ, quadratojugal.

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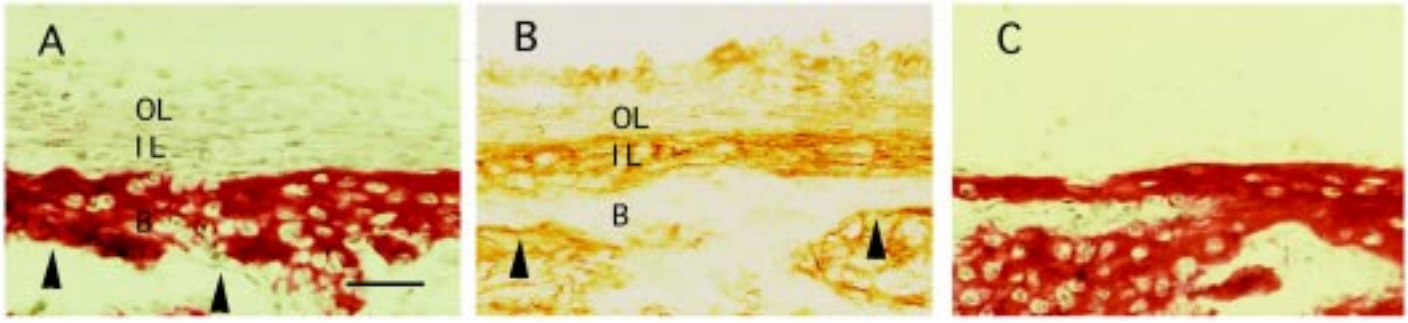

Fig. 1. Morphology of the quadratojugal periosteum and N-CAM expression. (A) *Morphology of the QJ. The periosteum consists of an outer layer (OL) and an inner layer (IL). The cells in the inner layer undergo osteogenic differentiation, secrete bony ECM and finally form bone (B) which is stained red by HBQ staining. Arrowheads indicate endosteal sites. Bar, 30 μm (for 1A-C).* **(B)** *N-CAM immunostaining of the QJ. N-CAM is positive in the inner cell layer (IL) of the periosteum. The outer layer (OL) and bone cells (B) embedded in bony ECM are N-CAM negative. Cells in endosteal sites are also N-CAM positive (arrow heads).* **(C)** *Morphology of the QJ after two enzymatic digestions, showing the efficacy of the treatment and complete removal of both OL and IL cell layers; cf. Fig. 1B.*

Because of the importance of chondrogenesis in developmental events such as limb and mandibular morphogenesis, molecular mechanisms of primary chondrogenesis have been under intensive investigation. It is now clear that cell adhesion molecules (CAMs) play an important role. One CAM documented to be involved in primary chondrogenesis and in morphogenesis of a number of organs, is neural cell adhesion molecule (N-CAM) (Widelitz *et al*., 1993; Tavella *et al*., 1994). N-CAM is a cell surface glycoprotein mediating cell-cell and/or cell-matrix interactions (Cunningham *et al*., 1987; Edelman *et al*., 1987). Three N-CAM isoforms (180, 140 and 120 kDa) are generated by differential splicing of a single gene (Murray *et al*., 1986). The 140 kDa N-CAM enhances chondrogenesis in micromass culture of limb mesenchyme (Widelitz *et al*., 1993). However, given that condensation and initiation of chondrogenic differentiation are coupled temporally in primary cartilage, whether N-CAM increases chondrogenesis by promoting mesenchymal condensation and/or by stimulating chondrogenic differentiation is unclear (Widelitz *et al*., 1993). We took advantage of the temporal separation of condensation and initial differentiation in secondary chondrogenesis to investigate the role of N-CAM in the two processes. We chose the avian quadratojugal (QJ) for this study.

N-CAM is expressed during osteogenesis of the QJ, but is down-regulated when periosteal cells switch to chondrogenesis *in vivo* (Fang and Hall, 1995). In the present study, we use several *in vitro* approaches to demonstrate that N-CAM is present during osteogenesis but not chondrogenesis in membrane bone periosteal cells *in vitro* and that secondary chondrogenesis can be initiated *in vitro* without an N-CAM signal. Hence, N-CAM seems to be involved in condensation but not in secondary chondrogenic differentiation. Furthermore, an overexpression assay using a plasmid containing the N-CAM gene revealed an inhibitory role of N-CAM on secondary chondrogenesis.

Results

N-CAM expression in the QJ shafts of chick embryos

To reveal N-CAM expression *in vivo*, QJ shafts from day 13 embryos were examined by immunolocalization with a monoclonal antibody recognizing all three isoforms of N-CAM (Frelinger and Rutishauser, 1986). N-CAM was detected in periosteal as well as endosteal sites at the bone core (Fig. 1A,B). The periosteum consisted of an N-CAM-negative outer layer and an N-CAM-positive inner layer. Two enzymatic digestions resulted in removal of both outer and inner periosteal layers from the shafts (Fig. 1C).

N-CAM expression of periosteal cells during chondrogenesis in vitro

Monolayer, clonal and agarose culture conditions were used to monitor changes of N-CAM expression in QJ periosteal cells under culture conditions favoring chondrogenesis.

Monolayer culture

We have previously shown that chondrogenesis occurs in QJ periosteal cells in monolayer culture at exceptionally low cell densities (Fang and Hall, 1996). Periosteal cells differentiated into typical chondrocytes characterized by pericellular refractile matrix, type II collagen expression, and alcian blue-positive extracellular matrix (ECM). To assess N-CAM expression, immunostaining was carried out after periosteal cells were plated in monolayer cultures. No N-CAM expression was found in culture fixed on day 1 as demonstrated by the absence of reactivity shown in Figure 2A. Although the cultures consisted of morphologically heterogeneous cell types, including fibroblast-like and polygonal cells, all cells were N-CAM-negative. N-CAM remained negative in monolayer cultures fixed at 3, 7 and 14 days. In cultures with growing chondrocyte colonies at day 14, N-CAM was also negative in the entire culture, including chondrocyte colonies and surrounding cells (Fig. 2B). Thus, in monolayer culture, N-CAM expression was shut down during the first day and was not reexpressed throughout the culture period.

Clonal culture

Since secondary chondrogenic differentiation can occur at exceptionally low cell density, we tested if periosteal cells at clonal culture can also undergo chondrogenesis. Periosteal cells were plated clonally in 96-well culture plates. After two weeks, most clones were composed of fibroblast-like cells and did not show a chondrocytic phenotype. However, some clones gave rise to chondrocytes as identified by morphology and type II collagen immunostaining (Fig. 2C). As in monolayer culture, chondrocytes often appeared from polygonal cell clones and were often mixed with polygonal cells. Immunohistochemistry showed that N-CAM was neither expressed in chondrocytes nor in surrounding cells (data not show).

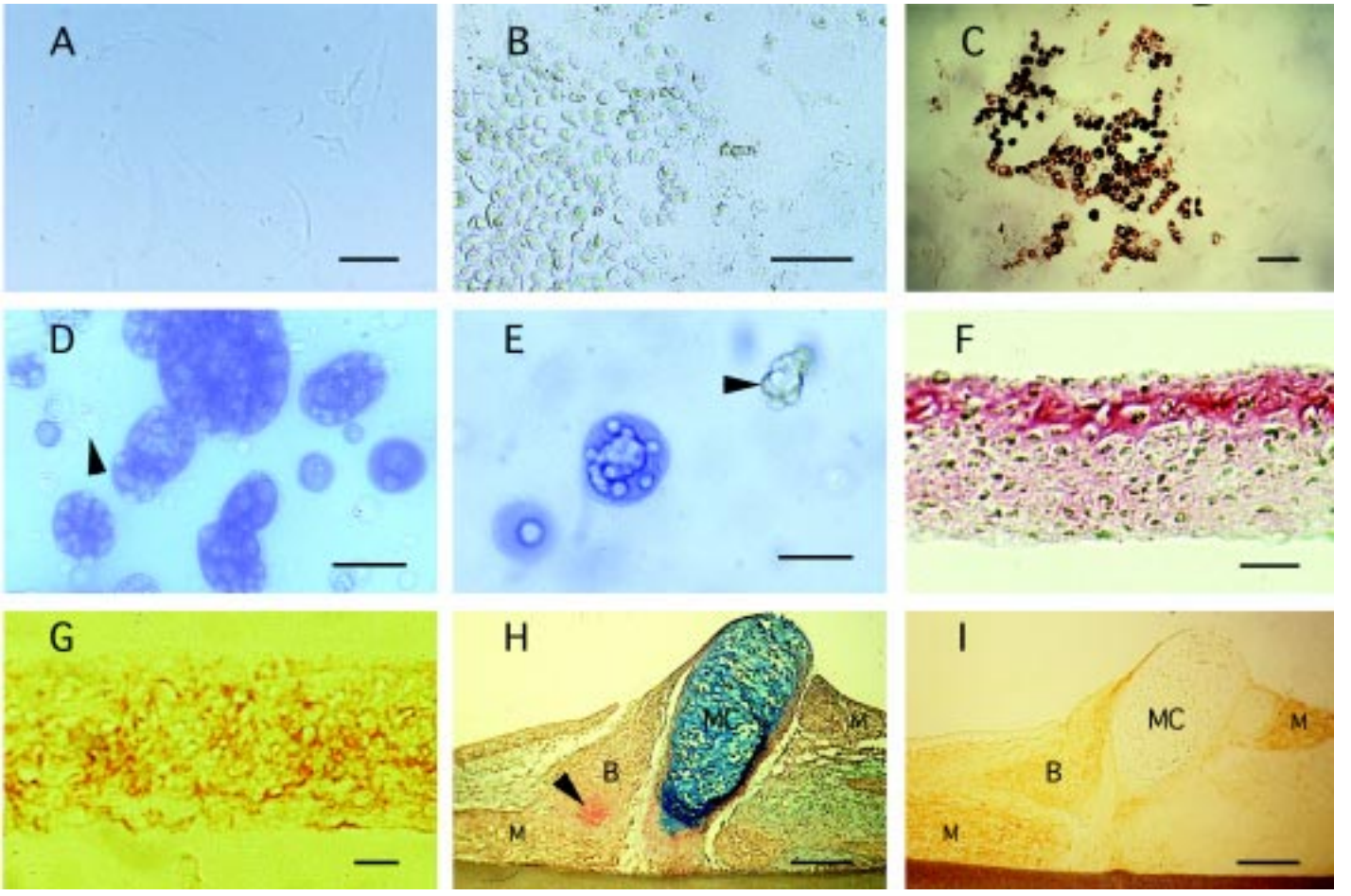

Fig. 2. N-CAM expression in various chondrogenic and osteogenic cultures. (A) *Immunohistochemistry for N-CAM in 24 h monolayer culture of QJ periosteal cells. Cells appear fibroblast-like or polygonal and show no N-CAM expression. No counterstain was applied to this section.* **(B)** *Immunohistochemistry for N-CAM in day 14 monolayer culture of periosteal cells. Chondrocytes are seen at the left; N-CAM expression above non-specific background is not detected in chondrocytes or surrounding cells.* **(C)** *Chondrogenesis of QJ periosteal cells in clonal culture, showing positive immunostaining for type II collagen.* **(D)** *Chondrogenesis of QJ periosteal cells in agarose culture. Chondrocyte colonies vary in size and are stained blue by alcian blue. Non-chondrogenic nodules (arrowhead) are small and unstained by alcian blue. A nuclear stain was not used because of the high background staining that would have been introduced.* **(E)** *Agarose culture with double staining for N-CAM and alcian blue, showing a single chondrocyte colony (left), a multiple chondrocyte colony (middle), and a non-chondrocyte colony (right). N-CAM is not expressed in chondrocytes but is seen in the non-chondrogenic colony (arrowhead).* **(F)** *Micromass culture of periosteal cells. HBQ staining shows bony ECM in the top cell layer.* **(G)** *N-CAM staining in micromass culture of periosteal cells, showing N-CAM expression in many cells.* **(H)** *Bone and cartilage formation in organ culture of the mandible. HBQ staining shows Meckel's cartilage (MC), condensed mesenchyme (M), and membrane bone (B). The arrow head indicates an osteogenic center.* **(I)** *N-CAM expression in organ culture of the mandible. N-CAM is expressed in condensed mesenchyme (M) and membrane bone (B), but not seen in chondrocytes in Meckel's cartilage (MC). (A-G) Bar (A-G), 40 μm; Bar (H-I), 80 μm.*

Agarose culture

Since suspension culture in agarose favors chondrocyte phenotype expression in dedifferentiated primary chondrocytes (Benya and Shaffer, 1982), we tested the chondrogenic potential of QJ periosteal cells in agarose culture. QJ periosteal cells can differentiate into chondrocytes when suspended in agarose gel. After two days in culture, some periosteal cells enlarged. They gradually secreted alcian blue-positive ECM and initiated mitosis. Chondrocyte colonies with multiple cells and abundant alcian blue-positive ECM first became visible at day 4. The numbers of chondrocyte colonies gradually increased over time. By two weeks, numerous chondrocyte colonies were seen after alcian blue staining. These colonies ranged from small – containing a single cell – to large, containing many cells (Fig. 2D). Thus, although chondrogenesis was normally associated with clusters of chondrocytes, single cells also expressed the chondrogenic phenotype. Immunostaining revealed that chondrocytes in agarose culture were N-CAM-negative (Fig. 2E). Even in chondrocyte colonies containing a single chondrocyte, that chondrocyte was N-CAM negative (Fig. 2E).

In addition, a second kind of cell colony arose in agarose culture. These colonies contained small cells which were compacted tightly. No alcian-blue positive ECM was associated with these colonies (Fig. 2D), indicating their non-chondrogenic nature. Unlike chondrogenic colonies, these compacted cell nodules, which do not exhibit a cartilage phenotype, express N-CAM (Fig. 2E).

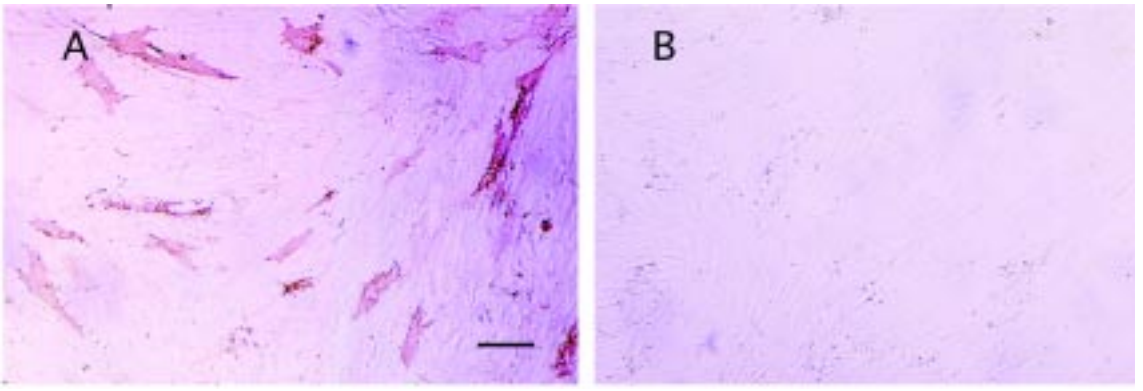

Fig. 3. Overexpression of N-CAM in periosteal cells by transfection. (A) *Immunostaining of N-CAM in periosteal cells transfected with pEC 1402 plasmid, showing localization of N-CAM in cultured periosteal cells.* **(B)** *Immunostaining of N-CAM in periosteal cells transfected with control plasmid. No N-CAM is detected. Bar, 40 μm.*

N-CAM expression in QJ periosteal cells in osteogenesis in vitro

Since N-CAM expression disappeared when periosteal cells were plated in monolayer culture and since N-CAM was not detected in cultures which supported chondrogenesis, we examined N-CAM expression in situations where osteogenesis occurred *in vitro*.

First, N-CAM immunostaining was conducted in histological sections of micromass cultures of QJ periosteal cells; as reported previously (Fang and Hall, 1996), QJ periosteal cells express an osteogenic-like phenotype in micromass culture. Immunostaining showed that N-CAM expression was retained in the periosteal cells in micromass culture (Fig. 2F,G).

Second, we examined N-CAM expression during mandibular bone formation of mandibles in organ culture. When mandibular processes from H.H. stages 23-24 embryos were cultured at the air-medium interface for seven days, osteogenic centers developed beside Meckel's cartilage. Mesenchymal cells condensed and became polygonal in shape. Then they gradually secreted bony ECM which was stained red by HBQ stain (Fig. 2H). This process is the same as intramembranous ossification *in vivo*. Immunostaining showed that N-CAM was expressed in osteogenic cells as well as in condensed mesenchymal cells (Fig. 2I). N-CAM expression declined when osteogenic cells became embedded in bony matrix. In contrast, chondrocytes in Meckel's cartilage were N-CAM negative (Fig. 2I).

Effect of N-CAM overexpression on chondrogenic differentiation in periosteal cells

To examine the physiological role of N-CAM on chondrogenic differentiation, plasmids encoding the whole chicken 140 kDa N-CAM were transfected into primary cultures of QJ periosteal cells using a liposomal transfection technique (the efficiency of an alternative approach – transfection mediated with viral vectors – varies with the availability of cell surface receptors such as integrins). With immunohistochemistry, N-CAM was detected on the surface of the cells transfected with N-CAM plasmid (Fig. 3A) but not on the cells transfected with a control plasmid that lacked the N-CAM sequence (Fig. 3B). N-CAM expression levels varied among cells. Some cells had strong positive staining on the cell surface and weak cytoplasmic staining, while others had weak cell surface and cytoplasmic staining. The percentage of positive cells was counted in trypsinized cells smeared on slides. About 6% were strongly positive and 37% weakly positive.

To assess the effect of N-CAM on chondrogenic differentiation, transfections were carried out in low density monolayer cultures ($5x10^3$ cells/ml) at day 6, a time immediately before initiation of chondrogenic differentiation. The experiment was conducted in three groups of cultured periosteal cells: i) transfected with N-CAM plasmid, ii) transfected with control plasmid, iii) not transfected with any plasmid (the transfection procedure was carried out without plasmid DNA). Chondrocyte colonies were identified based on immunolocalization of type II collagen in day 10 cultures; see Fang and Hall (1996) for figures of such colonies. Eight wells were counted for each groups. The average number of chondrocyte colonies in cultures transfected with pEC 1402 plasmid was significantly lower than in cultures transfected with control plasmid ($P<0.01$), or in non-transfected cultures ($P<0.01$), indicating an inhibitory effect of N-CAM on chondrogenic differentiation (Fig. 4).

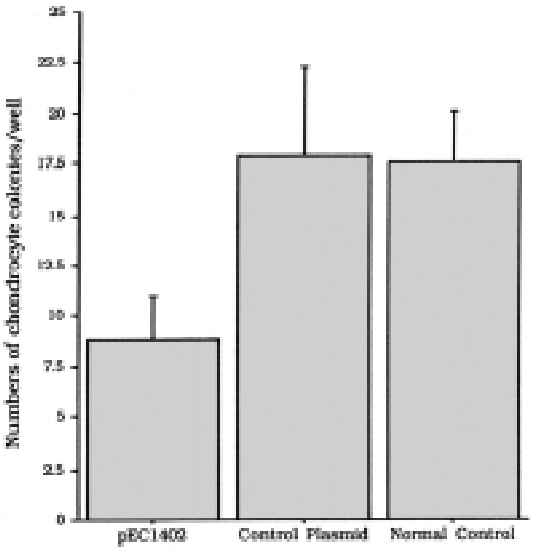

Fig. 4. Inhibitory effect of N-CAM overexpression on chondrogenesis. *Periosteal cells were plated in low density monolayer culture. Transfection was conducted in day 6 cultures with N-CAM plasmid (pEC 1402), control plasmid, or no plasmid (normal control). The numbers of chondrocyte colonies (means ± SD) were counted from 8 wells for each group in day 12 cultures based on type II collagen immunostaining . Student T test showed that there were significant differences in numbers of chondrocyte colonies between pEC 1402 and control plasmid groups ($P<0.01$) and between pEC 1402 and normal control groups ($P<0.01$).*

Discussion

This study demonstrates that N-CAM is expressed in osteogenic cells in various situations of membrane bone formation *in vitro*. However, N-CAM was not detected when periosteal cells underwent chondrogenesis *in vitro*. These results are consistent with and expand our previous studies that N-CAM is down-regulated during the switch from osteogenesis to secondary chondrogenesis in membrane bone periostea *in vivo* (Fang and Hall, 1995) and suggest an association of N-CAM expression with osteogenic but not with secondary chondrogenic differentiation.

Precartilaginous condensation is a critical step for development of cartilage from mesenchyme (Hall and Miyake, 1992, 1995). N-CAM is up-regulated during precartilaginous condensation of mesodermal (limb bud) mesenchyme (Chuong *et al.*, 1993; Widelitz *et al.*, 1993) and neural crest (mandible) mesenchyme (unpublished observation). Hence, N-CAM participates in primary cartilage formation in mesenchyme regardless of mesodermal or neural crest origins. In micromass culture of limb bud mesenchymal cells, numbers and sizes of cartilage nodules increased following overexpression of N-CAM but are reduced following exposure to N-CAM antibodies (Widelitz *et al.*, 1993), suggesting a stimulatory role of N-CAM on chondrogenesis. It was not clear from this study, however, whether N-CAM was required merely for cellular recruitment and aggregation in condensation, or whether N-CAM was also involved in the signal transduction required for initiation of chondrogenic differentiation. Our study shows that, although N-CAM is expressed in periosteal cells *in vivo,* it quickly disappears when the periosteal cells are plated in monolayer culture. Overt chondrogenic differentiation can take place in monolayer, clonal and suspension cultures of periosteal cells in the absence of N-CAM expression. This result indicates that secondary chondrogenesis can occur without participation of N-CAM.

Cellular condensation is often regarded as an absolute prerequisite for chondrogenesis from mesenchyme cells. This is true for primary cartilage formation *in vivo* and cartilage nodule formation from limb bud and mandibular mesenchyme in micromass culture. However, condensation and chondrogenic differentiation are separate events. During secondary chondrogenesis, precartilaginous condensation is not seen when periosteal cells switch from the osteogenic to the chondrogenic differentiation pathway. We have demonstrated that secondary chondrogenesis can occur in monolayer, clonal, and suspension cultures of periosteal cells in the absence of condensation. The formation of colonies with a single chondrocyte in agarose culture indicates that a periosteal cell can transform to a chondrogenic phenotype without undergoing mitosis and/or cell-cell interaction. Hence, chondrogenic differentiation can be independent from precartilaginous condensation. Condensation is probably mainly responsible for the overall patterning of the skeletal element, not for initiation of chondrogenic differentiation. Recent molecular evidence also shows that patterning and cell differentiation in skeletal tissues are achieved through different molecular pathways (Ducy *et al.*, 1997). Thus, molecules involved in precartilaginous condensation are not necessarily required for initiation of chondrogenic differentiation. Since N-CAM is expressed in precartilaginous condensation but absent during initiation of chondrogenic differentiation,

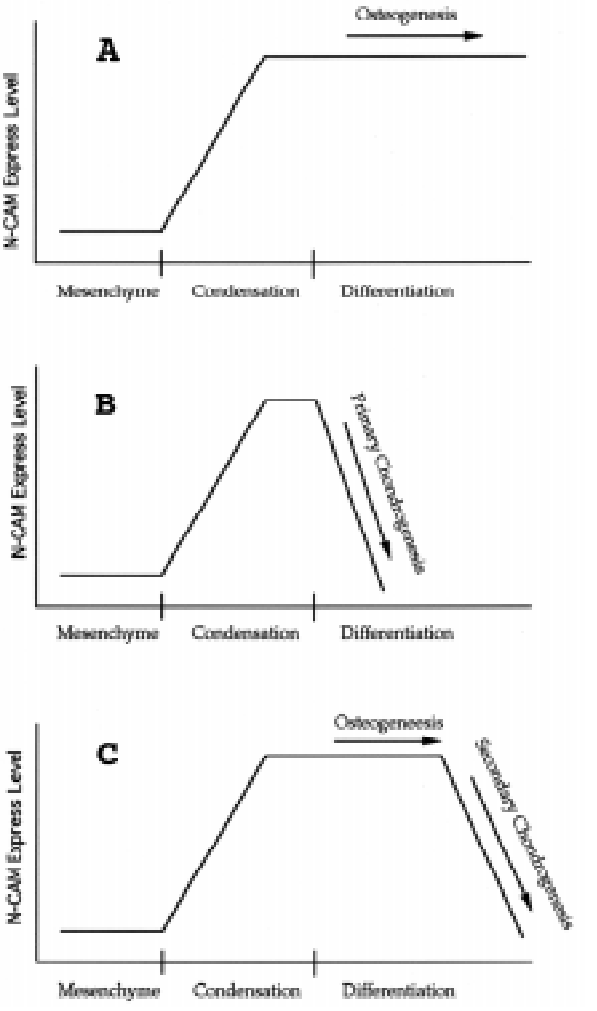

Fig. 5. Relationship between N-CAM expression, condensation and cell differentiation in osteogenesis (A) and in primary (B) and secondary (C) chondrogenesis. *N-CAM is up-regulated in mesenchymal condensation* **(A-C)**. *N-CAM expression remains during osteogenic differentiation* **(A)** *but is down-regulated during both primary and secondary chondrogenesis* **(B,C)**. *The pathways differ between primary and secondary chondrogenesis. Primary chondrogenesis arises directly from condensation mesenchyme while secondary chondrogenesis occurs after mesenchyme has committed to osteogenesis. N-CAM is down-regulated after condensation in primary chondrogenesis but with differentiation in secondary chondrogenesis.*

N-CAM is likely involved in condensation *per se* rather than as a signal for chondrogenic differentiation.

What then is the significance of the down-regulation of N-CAM seen during the switch from osteogenic to chondrogenic differentiation in secondary chondrogenesis? To answer this question, N-CAM overexpression tests were carried out. The effects of N-CAM overexpression on secondary chondrogenesis was assessed in low cell density monolayer culture of QJ periosteal cells. Overexpression of N-CAM before initiation of secondary chondrogenesis significantly reduced the number of chondrocyte colonies, i.e., N-CAM plays an inhibitory role on chondrogenic expression in QJ periosteal cells. This result is consistent with the transient expression pattern of N-CAM during secondary chondrogenesis in the QJ hook *in vivo* (Fang

and Hall, 1995) and N-CAM expression data *in vitro*. Since N-CAM prevents secondary chondrogenic differentiation, down regulation of N-CAM is therefore a prerequisite for secondary chondrogenesis in periostea of membrane bones.

Integrating our data with those from others, it seems that N-CAM plays different roles in development of different skeletal tissues. N-CAM expression in osteogenesis, primary chondrogenesis, and secondary chondrogenesis is schematically illustrated in Figure 5. How N-CAM may function during skeletal development is proposed below.

N-CAM is expressed during cellular condensation in both cartilage and membrane bone development (Fig. 5). Condensation is an essential cellular event in morphogenesis of many organs. N-CAM participates in many, if not all of those processes, as in the formations of feathers (Chuong and Edelman, 1985), kidney tubules (Crossin *et al.*, 1985), muscle (Knudsen *et al.*, 1990; Soler and Knudsen, 1991). Morphogenesis is regulated by pattern-regulatory genes such as *Hox* genes, which encode genetic information for position and shape of a particular organ element. The regulatory region of the N-CAM gene contains a homeodomain binding site (Hirsch *et al.*, 1990, 1991) and N-CAM gene expression can be regulated by some *Hox* genes (Jones *et al.*, 1992; Valarché *et al.*, 1993). Thus, N-CAM is probably a downstream target of pattern-determining genes and may directly cause cellular condensation by enhancing cell aggregation. In this regard, N-CAM may play a stimulatory role in both cartilage and membrane bone formation at the condensation stage by enhancing aggregation and recruitment of mesenchymal cells, resulting in more potential chondrogenic or osteogenic cells. This would explain why enhancement of N-CAM in mesenchyme increases cartilage formation and perturbation of N-CAM with antibodies decreases cartilage formation, as demonstrated previously (Chuong *et al.*, 1993; Widelitz *et al.*, 1993).

On the other hand, after condensation, the cells in precartilaginous and membrane bone condensations undergo further differentiation to express chondrogenic or osteogenic phenotypes. N-CAM expression differs at this stage between the two skeletal tissues: N-CAM persists in osteogenesis (Fig. 5A) but is down regulated as cells undergo chondrogenesis (Fig. 5B,C). This raises the possibility that N-CAM may play a role in further differentiation of skeletal cells. This is supported by down-regulation of N-CAM during secondary cartilage development in the QJ hook (Fang and Hall, 1995). More importantly, our data indicated that N-CAM might play an inhibitory role on chondrogenesis in monolayer culture of QJ periosteal cells. Thus, N-CAM may influence skeletal cell differentiation by preventing expression of the chondrocyte phenotype; down regulation of N-CAM is a pre-requirement for alteration of differentiation pathways of periosteal cells from osteogenesis to chondrogenesis. When function-perturbing antibodies against N-CAM become available it will be possible to test directly the ability of N-CAM to promote chondrogenesis in high density cultures.

In summary, N-CAM plays opposite roles at two development stages of chondrogenesis, stimulating chondrogenesis by promoting precartilaginous condensation, but inhibiting chondrogenic phenotypic expression in further differentiation. During osteogenesis, N-CAM expression is maintained until mature osteocytes form. Down-regulation of N-CAM is a prerequisite when periosteal cells switch to chondrogenesis.

Materials and Methods

Chick embryos and periosteal cells of the quadratojugals

Fertilized white leghorn chicken eggs from Cook's Hatchery (Truro, NS) were incubated in a humidified incubator at 36±0.5°C. Quadratojugal periosteal cells were prepared as described previously (Fang and Hall, 1996). Briefly, QJs were aseptically dissected from day 13 embryos and QJ hooks were removed. The QJ shafts were rinsed, pooled and digested in 200 units/ml collagenase type IA (Sigma, St. Louis, MO) and 0.5% trypsin (BDH, Canada) in PBS with stirring in a vortex at room temperature for 20 min. The QJ shafts were transferred to fresh enzyme solution and digested for another 30 min. Cells released from the second digestion were filtered through two layers of gauze, washed with PBS, and resuspended in Ham's F-12+BGJb (3:1) media containing 10% fetal bovine serum (all from GIBCO BRL, Burlington, ON). Cell numbers were counted with a hemocytometer. Obviously, it is not possible to obtain preparations that consist of 100% outer (fibroblastic) or 100% inner (osteogenic) cells. However, the absence of osteogenesis from cells of the fibroblastic layer indicates that a functional separation of the two layers was achieved.

Monolayer culture

The periosteal cells were plated in 24-well culture plates at $1x10^4$ cells/ml (day 0). Culture medium consisted of Ham's F-12+BGJb (3:1) with 10% FBS, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in 37°C with 5% CO_2 and fixed with periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) at days 1, 3, 7, or 14 for 10 min.

Clonal culture

To grow single cell in micro wells, the periosteal cell suspension was serially diluted to a final concentration of 20 cells/ml. Then, 100 μl of cells were transferred into each well of 96-well plates containing 100 μl of conditioned medium. The conditioned medium was collected from monolayer culture of QJ periosteal cells and filtered through a 0.25 μm Millipore filter. The culture medium was the same as in monolayer culture. Individual wells were carefully observed under an inverted microscope and those wells containing single cell were marked. The plates were incubated in 5% CO_2 at 37°C for up to 14 days.

Suspension culture of periosteal cells in agarose gel

Periosteal cells were resuspended in double strength (2x) DMEM medium (Gibco BRL, Burlington, ON) at $2x10^5$ cells/ml. Cell suspension was mixed with an equal volume of 1% low gelling temperature agarose (type VII, Sigma, St. Louis, MO) prepared in sterile dH_2O to yield a final concentration of $1x10^5$ cells/ml. The cell-agarose mixtures were pipetted as 50 μl drops onto 35 or 60 mm dishes which had been pre-coated with 1% high gelling temperature agarose (type V, Sigma, St. Louis, MO). The culture dishes were chilled at 4°C for 15 min to solidify the agarose-cell drops. Then complete medium (2 ml to each 35 mm dish well and 4 ml to each 60 mm dish well) was added to each well. The medium was the same as above. The cultures were incubated at 37°C in a 5% CO_2 incubator for 14 days and fixed with PLP fixative overnight. Use of DMEM medium followed the original protocol of Benya and Shaffer (1982). Substitution of $F12+BGJ_b$ for DHEM did not affect the results.

Micromass culture of QJ periosteal cells

Periosteal cells were adjusted to a concentration of $2x10^7$ cells per ml with culture medium. One drop (10 μl, or $2x10^5$ cells) of cell suspension was placed into wells of 24-well culture plates. After 2 h in a CO_2 incubator, the cells were flooded with 1 ml culture medium (Ham's F-12+BGJb (3:1), containing 10% FBS, 1 mM L-glutamine, and 150 μg/ml L-ascorbic acid). The medium was changed every 2-3 days. Morphology of the cultures was examined daily under an inverted microscope. After 14 days, the micromasses were peeled off the culture plates and processed for histology and immunohistochemistry.

Organ cultures of mandibles

The mandibular processes of chick embryos at H.H. (Hamburger and Hamilton, 1951) stages 23-24 were aseptically dissected under a dissecting microscope, rinsed with PBS twice and placed on black Millipore filters cut into about 0.5x0.5 cm pieces. The intact mandibles on the black filters were cultured at the air/medium interface on stainless steel grids in 35 mm tissue culture dishes in a 5% CO_2 incubator. Each dish contained 2 ml medium of Ham's F-12 and BGJb (3:1) containing 10% FBS, 150 μg/ml ascorbic acid, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The medium was changed once at day 4. The mandibles were collected at day 7, fixed in 70% ethanol or PLP fixative overnight, and processed for histology and immunohistochemistry.

Plasmids and transfection

pEC 1402 plasmid (kindly provided by Dr. B.A. Murray, University of California, Irvine, CA., see Edelman *et al*., 1987), which contains the whole length 140 kDa N-CAM gene under control of the SV40 early promoter, was used for overexpression of N-CAM. A control plasmid was constructed by deleting the N-CAM sequence from the pEC 1402 plasmid by digesting pEC 1402 with BamH I and rejoined by T-4 DNA ligase. Both pEC 1402 and control plasmid were purified either by double CsCl gradient ultracentrifugation or a Qiagen (Santa Clarita, Ca) large-scale plasmid DNA purification kit.

To overexpress N-CAM, transfection was performed using LipofectAMINE reagents from GIBCO, BRL (Burlington, ON). Briefly, 2 μg of pEC 1402 DNA or control plasmid DNA was mixed with 5 ml LipofectAMINE Reagent in F-12+BGJb (3:1) medium without FBS for 30 min. Then 800 μl of the same medium was added to make 1000 μl of transfection solution. Transfections were conducted by incubating periosteal cells with the transfection solution for 6 h. For assessment of transfection efficiency, periosteal cells were plated at $2X10^5$ /ml in 24-well plates (day 0), transfected by day 2 and fixed with the PLP fixative for immunohistochemistry by day 4. For evaluation of phenotypical changes, periosteal cells were plated at $5x10^3$ /ml in 24-well plated (day 0), transfected by day 6, and fixed for type II collagen immunohistochemistry by day 10.

Immunohistochemistry and histology

Monoclonal antibodies against N-CAM (5e) and chicken type II collagen (II-II6B3) were obtained from the Developmental Studies Hybridoma Bank. The avidin-biotin-peroxidase complex (ABC) immunohistochemistry was modified from Hsu *et al.* (1981). Non-specific binding sites were blocked with normal goat serum. The cultures were first incubated with primary antibodies for 2 h at room temperature. After PBS wash, the specimens were incubated with 1:200 diluted biotinylated goat anti-mouse IgG (GIBCO BRL, Burlington, ON) for 30 min. The samples were rinsed and incubated with the avidin-biotin-peroxidase complex (VECTASTAIN elite ABC kit, Vector Laboratories, Buringame, CA) for 30 min. Finally, a brown precipitate was produced with 3,3'-diaminobenzidine (DAB) in 0.05M, pH 7.6 Tris-HCl buffer containing 4 μl of 30% hydrogen peroxide (H2O2).

Histological sections of QJs and mandibles were prepared as described previously (Fang and Hall, 1995). Bone tissue and cartilage were discriminated using the HBQ stain (Hall, 1986).

Alcian blue stain

Cultures were fixed with in 70% ethanol, rinsed in 3% glacial acetic acid (pH 1), and stained in 0.5% alcian blue in 3% glacial acetic acid (pH 1) for 1 h. After rinsing in 3% glacial acetic acid and then PBS, the cultures were examined and photographed with an inverted microscope.

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