Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis

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SUMMARY

Cell adhesion molecules have been shown to be important mediators of morphogenesis and pattern formation. In this study, we have shown that N-cadherin is expressed in a specific spatiotemporal manner in the developing limb bud during chondrogenesis in vivo and in cultured limb mesenchyme in vitro. The time period of maximal expression of N-cadherin corresponds to the period of active cellular condensation, an event believed to be a necessary prerequisite for chondrogenic differentiation. To directly assess the functional involvement of N-cadherin in cellular condensation, we have examined the effects of perturbing N-cadherin activity on both cell aggregation and chondrogenesis using NCD-2, a rat monoclonal antibody directed against the binding region of N-cadherin. Non-immune rat IgG was used as a control. Our results show that functional N-cadherin is necessary for chondrogenesis to proceed both in vivo and in vitro. Limb mesenchymal cells exhibited characteristic Ca\(^{2+}\)-dependent cell aggregation in suspension, which was inhibited in the presence of exogenous NCD-2. In micromass cultures of limb mesenchymal cells, NCD-2 inhibited overt chondrogenesis in a dose-dependent manner. Furthermore, NCD-2 inhibition of chondrogenesis in micromass cultures was time-dependent, suggesting that N-cadherin is crucially involved during the latter half of the first 24 hours of culture, a time period most likely corresponding to active cellular condensation. NCD-2 also significantly influenced limb development when injected into embryonic limb buds in vivo. In addition to significant inhibition of chondrogenesis and developmental delays, gross developmental deformities and perturbation of overall pattern formation were also observed. Taken together, these results demonstrate that N-cadherin is functionally required in mediating the cell-cell interactions among mesenchymal cells important for chondrogenesis in micromass culture in vitro and in the intact limb bud in vivo.

Key words: cell-cell interaction, morphogenesis, extracellular matrix, limb mesenchyme, skeletogenesis, cellular condensation

INTRODUCTION

Cell-cell adhesion and the events that subsequently take place between interacting cells, play important functional roles in biological processes (Grunwald, 1991). Cell adhesion molecules are recognized as being important mediators of both embryonic developmental and morphogenetic events, as well as stabilizers of adult tissue structures. The two main families of cell adhesion molecules are the Ca\(^{2+}\)-dependent cadherin superfamily, and the Ca\(^{2+}\)-independent immunoglobulin superfamily (CAMs; Edelman and Crossin, 1991; Takeichi, 1990, 1991; Geiger and Ayalon, 1992). Members of both these families of cell adhesion molecules mediate cell-cell adhesion in a homophilic manner. The classical cadherins, such as N-cadherin (Hatta et al., 1988) and E-cadherin (Nagafuchi et al., 1987), are transmembrane glycoproteins of about 120×10^3\( M_r \) that require interaction with the cytoskeleton in order to mediate extracellular binding. Such interactions are likely to involve the catenins, a group of at least three proteins (\( \alpha \), \( \beta \), and \( \gamma \)) which both co-localize and co-immunoprecipitate with solubilized cadherins (Ozawa et al., 1989; Peifer et al., 1992). The three catenins, \( \alpha \), \( \beta \), and \( \gamma \), are closely related to vinculin, armadillo, and plakoglobin, respectively.

The expression of a cell adhesion molecule during development usually coincides with substantial morphogenetic events. Chondrogenesis in the embryonic chick limb bud represents an interesting model system, in view of the early cellular condensation phase (Fell and Canti, 1934; Thorogood and Hinchliffe, 1975; Ede, 1983; Newman et al., 1985). At around stage 23/24 of development (Hamburger and Hamilton, 1951), mesenchymal cells of the limb bud core come into close apposition with one another (i.e. condensation) and subsequently differentiate into the first cartilaginous structure in the limb. This is accompanied by the synthesis and secretion of collagen type II and the protein core of the large chondroitin-rich proteoglycan known as aggrecan. The close apposition of the mesenchymal cells could result from or facilitate the formation of cell-cell interactions necessary for chondrogenesis. Interestingly, Widelitz et al. (1993) recently reported that N-CAM, a neural Ca\(^{2+}\)-independent cell adhesion molecule, was
expressed in condensing aggregates of chick limb mesenchymal cells both in vivo, and cultured as micromass in vitro. In addition, these investigators demonstrated that inhibiting N-CAM mediated cell-cell adhesion with specific antibodies against N-CAM, resulted in the inhibition of overt chondrogenesis in vitro.

In considering how other cell adhesion processes may be functionally involved in limb bud mesenchymal condensation and chondrogenesis, it is noteworthy that San Antonio and Tuan (1986) previously demonstrated that exogenous Ca\textsuperscript{2+} significantly stimulates chondrogenesis in limb mesenchymal micromass cultures. This effect acts predominantly during the first 24 hours of culture, when mesenchymal cells are actively condensing (San Antonio and Tuan, 1986; Evans and Tuan, 1988; Tuan, 1991). Additionally, Bee and Von der Mark (1990) have also described a Ca\textsuperscript{2+}-dependent cell adhesion phenomenon in isolated limb mesenchymal cells. These observations suggest that Ca\textsuperscript{2+} may affect or influence the cell-cell interactions in mesenchymal condensation, perhaps involving cadherins.

In this study, we have investigated the possible involvement of cadherins in chondrogenesis, particularly during mesenchymal condensation. Specifically, we have focused on neural cadherin (N-cadherin) because of its transient expression in various mesenchymal populations during embryonic development (Hatta and Takeichi, 1986; Hatta et al., 1987). To test this postulate, we have first identified N-cadherin expression in limb mesenchyme, and secondly, perturbed N-cadherin mediated cell-cell adhesion in embryonic limbs in vivo and in cultured limb mesenchyme in vitro by employing specific monoclonal antibodies that recognize an epitope in the binding region of N-cadherin. Our results show that N-cadherin is expressed in populations of mesenchymal cells that are undergoing active cellular condensation, and is absent from differentiated cartilage. In addition, perturbation of N-cadherin binding function significantly inhibits both limb mesenchymal Ca\textsuperscript{2+}-dependent aggregation, and chondrogenesis in vitro and in vivo.

**MATERIALS AND METHODS**

**Chick embryos and shell-less embryo culture**

Fertilized White Leghorn chicken eggs (Truslow Farms, Chestertown, MD) were incubated at 99°F in a humidified egg incubator for the desired period of time. For in vivo perturbation of N-cadherin, shell-less, cultured chick embryos were used as previously described by Tuan (1980). Briefly, after 3 days of incubation in ovo, chick embryos were placed without the eggshell into ringstands lined with plastic kitchen wrap, covered with a Petri dish lid, and maintained in a humidified incubator at 37.5°C with constant air flow. This method permitted easy access to the limb bud and continuous observation of development.

**Limb mesenchymal cell isolation and differential protease sensitivity**

This isolation procedure was adapted from that described by Ahrens et al. (1977) and modified by San Antonio and Tuan (1986). Briefly, limb buds were dissected from chick embryos at stage 23/24, cut, and enzymatically dissociated in Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free saline G (CMFSG) containing 0.1% trypsin (Type II, Sigma) and 0.1% collagenase (Worthington). Isolated cells were resuspended in CMFSG + 10% fetal calf serum (FCS), counted with a hemocytometer in the presence of trypsin blue (over 95% dye exclusion), adjusted to the appropriate plating density (between 10-12x10\textsuperscript{6} cells/ml), plated in a 100 µl drop per well on 6 well tissue culture dishes (Corning), allowed to attach, and then covered with 3 ml of culture medium (CM) containing Ham’s F-12, 10% FCS, 0.2% chick embryo extract, and 1% penicillin-streptomycin (CM). These cells were allowed to recover in culture for 15-18 hours, and then collectively dissociated with 0.01% trypsin in the presence of either 1.5 mM CaCl\textsubscript{2} (TC) or 1 mM EGTA (TE) as described by Takeichi (1977). Isolated cells were resuspended in HCMFSG and counted. In certain experiments, the HCMFSG was supplemented with additional reagents as described below.

**Analysis of cell aggregation**

TC- (or TE-) treated cells were resuspended in HCMFSG (±2 mM CaCl\textsubscript{2}), and supplemented with either control rat IgG (Sigma) or purified NCD-2 antibodies. To initiate cell aggregation, 2-2.5x10\textsuperscript{5} cells were placed into the well of a 24-well plate previously coated with 1% bovine serum albumin (BSA), covered with 1 ml of HCMFSG with or without the addition of antibodies and 2 mM CaCl\textsubscript{2}, and then incubated at 37°C on a gyrating shaker at 80-100 rpm. An aliquot from each well was removed at various time points and counted with a Coulter Counter (Model ZM) equipped with a 100 µm aperture. The degree of aggregation was determined by dividing the number of particles counted at each time point by the number of particles at time zero.

**Micromass cultures**

A 10 µl drop of TC cells at 8-15x10\textsuperscript{5} cells/ml was plated per well on a 24 well plate and allowed to attach for 1-2 hours at 37°C and 5% CO\textsubscript{2}, after which 1 ml of CM was added to each well. For most experiments, the medium was replaced with fresh medium every 24 hours. The effects of various agents were tested by direct additions to the culture medium, and cells cultured for the desired period of time. For some experiments, FCS was heated at 56°C for 30 minutes prior to use to inactivate the complement components. Chondrogenesis was quantified based on sulfate incorporation ([\textsuperscript{35}S]Na\textsubscript{2}SO\textsubscript{4}, 2.5 Ci/ml, ICN) and staining with Alcian blue 8-GX (Sigma) at pH 1.0 (San Antonio and Tuan, 1986).

**N-cadherin monoclonal antibodies**

The NCD-2 rat hybridoma, a kind gift of Dr M. Takeichi, produces a monoclonal antibody that specifically recognizes the extracellular binding region of N-cadherin and effectively neutralizes its normal binding functions (Hatta and Takeichi, 1986). IgG antibodies were isolated from the NCD-2 culture supernatant by (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation and bulk phase DEAE-Sephadex ion-exchange chromatography (Harlow and Lane, 1988), diluted to known concentrations, and stored at −20°C until use.

**Immunohistochemical localization of N-cadherin**

Chick embryonic limb buds

Limbs buds were excised from embryos at various stages of development (stages 18-30), fixed in 4% paraformaldehyde-PBS at 4°C for 1-2 hours, rinsed in Tris-buffered saline with 2 mM CaCl\textsubscript{2} (TBS/Ca) and then frozen-embedded in Tissue-Tek OCT compound (Miles). Cryosections (8 µm) were placed onto Neoprene coated slides for immunohistochemical staining (Lagunowich and Grunwald, 1989). Briefly, after blocking with 5% normal goat serum (NGS) and washing, the sections were incubated with unlabeled NCD-2 culture medium for 30 minutes at room temperature. Controls were incubated in either unconditioned culture medium or TBS/Ca. After incubation, the slides were washed in TBS/Ca, blocked a second time in 5% NGS, and then incubated with fluorescein-conjugated goat anti-rat antibody (Zymed) at a dilution of 1:1000 for 30 minutes at room temperature. The sections were viewed with an Olympus BH-2 microscope equipped with epifluorescence and Nomarski differential interference optics.
Micromass limb mesenchyme cultures
Micromass cultures plated at 11×10^6 cells/ml on 8-chamber glass Lab-Tek slides (Nunc) were processed for immunohistochemical staining as described above with the following modifications: blocking with 10% NGS, incubation with purified NCD-2 IgG (5 mg/ml) at 50 μg/ml, and secondary antibody at a dilution of 1:4000.

Immunoblot analysis of N-cadherin
The micromass cell culture samples were solubilized in 60 mM Tris (pH 6.8) and 2% SDS and protein concentration determined (micro BCA kit, Pierce Chemical). Samples were adjusted to equal protein concentrations in a reducing sample buffer and analyzed by electrophoresis in a 7.5% polyacrylamide SDS gel. The proteins were electrophoretically transferred to nitrocellulose and incubated with purified NCD-2 antibodies (50 μg/ml) followed by biotin-conjugated goat anti-rat antibody (Zymed) at a dilution of 1:500. After washing, the blots were incubated with streptavidin-alkaline phosphatase and developed histochemically with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Injection of limb bud with NCD-2 antibodies in vivo
The limb buds of shell-less chick embryos at stages 22-24 were injected with antibodies. To visualize the efficacy of injection, the vital dye, Nile Blue sulfate, was added to solutions of both antibodies used, control rat IgG and NCD-2 (10 mg/protein/ml). Because the embryos were always situated on top of the yolk with mostly only its right side exposed, and it was undesirable to disrupt any vital embryonic vasculature, only one of the limbs, either the fore- or hindlimb was injected with NCD-2, and the other limb with rat IgG at the same concentration. The injections were carried out using a pulled 10 μl capillary tube attached to a microsyringe (Drummond), mounted on a micromanipulator. The volume injected into the limbs was typically between 0.5-1.0 μl (i.e. 5-10 μg of antibodies), and each embryo always received the same volume in each of its limbs. After the injections were completed, the embryos were placed back into the incubator and allowed to develop, uninterrupted.

Whole-mount Alcian blue staining of chick embryos
Two days after injection, the embryos were removed and fixed in PBS-buffered 10% formalin and subjected to whole-mount Alcian blue staining (Kimmel and Trammell, 1981). Briefly, the embryos were stained in acid-alcohol Alcian blue for 17 hours, cleared and macerated in 2% KOH, hardened in 50% glycerin, and stored in 100% glycerin. The limbs were then removed from the embryos and viewed and photographed using a Wild stereo microscope. All comparisons were made between the injected limb and the contralateral, uninjected limb. In addition, the rat IgG injected limbs served as both a control for possible effects due to the injection buffer or the presence of rat IgG, as compared to the contralateral, uninjected limb.

RESULTS

Immunohistochemical localization of N-cadherin expression in limb bud in situ and in micromass cultures in vitro
Limbs buds from various stages of chick embryos, from prechondrogenic to cartilaginous stages, were examined for the presence of N-cadherin by indirect immunofluorescence. N-cadherin was detected as early as stage 17/18, shortly after limb bud formation and was found to localize in a sparsely scattered pattern around the center of the limb bud (data not shown). At stage 24/25, when cellular condensation was clearly evident, N-cadherin was detected at high levels in the central, aggregating region of the limb bud (Fig. 1A,B). As the limb continued to develop, the condensed, central region began to lose N-cadherin protein expression, while the peripheral mesenchyme began to express N-cadherin. By stage 29/30, mature cartilage was visible and clearly displayed no immunoreactivity for N-cadherin, while the surrounding dense mesenchyme continued to show a high level of N-cadherin expression (Fig. 1C,D). It is noteworthy that some of these N-cadherin-positive cells would eventually contribute to the expanding cartilaginous core by appositional growth.

In micromass cultures in vitro, mesenchymal cells aggregate eventually to form discrete, cartilaginous nodules, separated by fibroblasts and myocytes (Ahrens et al., 1977). Immunohistochemistry showed that by 12 hours in culture, N-cadherin was expressed in aggregating (or condensing) regions within the culture, and the surrounding cells displayed no evident signal (data not shown). Expression of N-cadherin in the condensing mesenchyme intensified as a function of culture time and was maximal at around 18 hours (Fig. 1E,F), with the pattern of expression remaining essentially unchanged. During these early stages of in vitro culture, cells were seen to aggregate actively, and the aggregates would eventually give rise to differentiated, cartilaginous nodules, which did not express N-cadherin. The expression and distribution pattern of N-cadherin in micromass cultures in vitro was thus reminiscent of that in the developing limb bud in situ.

NCD-2 interferes with Ca^{2+}-dependent cell aggregation
To test the hypothesis that Ca^{2+} stimulates chondrogenesis by promoting mesenchymal cellular aggregation and condensation, we carried out cell adhesion studies employing the experimental design first described by Takeichi (1977). Limb bud mesenchymal cells were first allowed to recover from initial dissociation and isolation for 15-18 hours in culture, and were then dissociated by mild treatment with trypsin in the presence of 1.5 mM Ca^{2+} (TC) or 1 mM EGTA (TE). It has been shown (Takeichi, 1977; Urushihara et al., 1979) that TE treatment cleaves all cell surface cadherins and renders them non-functional, whereas TC treatment leaves cadherins intact and functional. The TC and TE dissociated cells were subsequently placed in suspension cultures and the kinetics of cell aggregation was analyzed. As shown in Fig. 2, TC cells readily aggregated in the presence of Ca^{2+} and control rat IgG antibodies, but displayed minimal aggregation in the absence of Ca^{2+} (Note: the baseline aggregation was most likely due to artificial adhesion caused by cellular debris and DNA derived from necrotic cells and/or mediated by Ca^{2+}-independent mechanisms). Characteristic of cells capable of Ca^{2+}-dependent cell adhesion, TE treatment substantially lowered their ability to aggregate, the level being similar with or without 2 mM Ca^{2+} (data not shown). This adhesion profile strongly suggests that the limb mesenchymal cells express cell surface cadherins and that they are functional in mediating cell-cell interactions in a Ca^{2+}-dependent manner. This was confirmed by immunoblot analysis (Fig. 2, inset), showing intact N-cadherin protein in TC cells immediately following proteolytic isolation, but not in TE cells. To conclusively determine whether N-cadherin was indeed mediating this aggregation event, we tested the effect of NCD-2 (see Materials and methods) on the aggregation of TC cells in suspension. As illustrated in Fig. 2, when NCD-2 was added at the same concentration as the control...
antibodies in the presence of the same concentration of Ca\(^{2+}\), aggregation of the TC cells was significantly perturbed. It should be noted that NCD-2 inhibition of TC cell aggregation was less than complete, i.e. to the same level as the Ca\(^{2+}\) deficient group. One explanation is that Ca\(^{2+}\)-independent cell adhesion molecules were present and functional on the surfaces of these cells; another possibility is that the NCD-2 antibodies did not completely obliterate N-cadherin action, perhaps because of sub-optimal stoichiometry.

**NCD-2 inhibits chondrogenesis in vitro**

To test whether NCD-2 interference of cell aggregation or adhesion could affect subsequent chondrogenic differentiation, NCD-2 was added at various concentrations to the culture medium of TC cells plated in micromass cultures for the first 24 hours of incubation, corresponding to the time period in which Ca\(^{2+}\) exerted its maximal effect (San Antonio and Tuan, 1986; Tuan, 1991). After 72 hours of incubation, the cultures treated with non-immune control rat IgG antibodies demonstrated roughly the same number of Alcian blue stained cartilaginous nodules, regardless of the concentration of antibody (Fig. 3A). However, increasing concentrations of NCD-2 significantly perturbed nodule formation in a dose-dependent manner in these micromass cultures (Fig. 3A). This profile of NCD-2 dose-dependent inhibition was also observed when chondrogenesis was measured in terms of \[^{35}S\]sulfate incor-

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**Fig. 1.** Immunohistochemical detection and localization of N-cadherin in the chick embryonic limb bud in situ and in micromass culture of limb mesenchyme in vitro. (A,C,E) Immunofluorescent staining of N-cadherin in limb bud cryosections (A,C) and in fixed micromass cultures (E). (B,D,F) Corresponding views of the sections using Nomarski differential interference optics. (A,B) Stage 24/25 limb sectioned transversely, showing prominent expression of N-cadherin in the condensing mesenchymal core region (arrows). The epithelium (E) demarcates the edge of the limb bud. (C,D) Stage 29/30 limb sectioned longitudinally, revealing mature cartilage (C) which was negative for N-cadherin expression, and dense mesenchyme (M) which was positive for N-cadherin expression. Scale bar, 100 \(\mu\)m for A-D. (E,F) Micromass culture 18 hours into incubation, displaying intense N-cadherin staining in a condensing aggregate of mesenchymal cells (arrow). The immediate surrounding mesenchyme was slightly positive and would most likely be incorporated into the cartilaginous core, while the mesenchyme located further away remained negative for N-cadherin expression. Scale bar, 50 \(\mu\)m for E and F.
Fig. 2. NCD-2 inhibition of Ca\textsuperscript{2+}-dependent aggregation of TC cells in suspension culture. Recovered limb mesenchymal cells were dissociated by the TC method (see Materials and methods) and placed into suspension cultures either with (+Ca) or without (–Ca) 2 mM CaCl\textsubscript{2} in the medium. The cultures with 2 mM Ca\textsuperscript{2+} were supplemented with either 300 μg/ml control rat IgG antibodies (Rat IgG) or 300 μg/ml NCD-2 antibodies (NCD-2). TC cells only aggregated to a minimal extent in the absence of Ca\textsuperscript{2+} (–Ca) as determined by the percentage of single cells remaining in culture at different time points. In the presence of 2 mM Ca\textsuperscript{2+} and control antibodies (+Ca, Rat IgG), the TC cells aggregated in a typical Ca\textsuperscript{2+}-dependent manner. However, in the presence of 2 mM Ca\textsuperscript{2+} and NCD-2 antibodies (+Ca, NCD-2), the TC cells were significantly perturbed in their ability to aggregate. Values represent the mean ± s.d. of 3 cultures. * Denotes statistical significance (P<0.05) compared to the +Ca, Rat IgG group. (Inset) Western immunoblot detection of N-cadherin in TC and TE cells immediately after isolation. Protein load was 35 μg/lane in the SDS-PAGE. Intact N-cadherin was detected in TC cells, whereas TE cells contained no detectable N-cadherin.

Table 1. Effects on limb chondrogenesis and development by NCD-2 antibody injection into chick embryonic limb buds in vivo

<table>
<thead>
<tr>
<th>Limb</th>
<th>No. of injected limbs</th>
<th>No effect* (% of total)</th>
<th>Slight-moderate delay* (% of total)</th>
<th>Moderate-severe delay* (% of total)</th>
<th>Gross deformity* (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wing (rat IgG)</td>
<td>21</td>
<td>11 (52.4)</td>
<td>9 (42.9)</td>
<td>1 (4.8)</td>
<td>(0)</td>
</tr>
<tr>
<td>Wing (NCD-2)</td>
<td>27</td>
<td>7 (25.9)</td>
<td>8 (29.6)</td>
<td>5 (18.5)</td>
<td>(25.9)</td>
</tr>
<tr>
<td>Hindlimb (rat IgG)</td>
<td>26</td>
<td>18 (69.2)</td>
<td>6 (23.0)</td>
<td>2 (7.7)</td>
<td>(0)</td>
</tr>
<tr>
<td>Hindlimb (NCD-2)</td>
<td>22</td>
<td>6 (27.3)</td>
<td>6 (27.3)</td>
<td>3 (13.6)</td>
<td>(31.8)</td>
</tr>
</tbody>
</table>

*The effects on limb chondrogenesis and development are categorized as shown in Fig. 5.
fore- and hind-limbs of these embryos. As seen in Table 1, the overwhelming majority of limbs injected with control rat IgG antibody displayed either normal development, or only slight-to-moderate developmental delays, as compared to the contralateral, un.injected control limb. However, both the forelimbs and hindlimbs that were injected with NCD-2 showed moderate-to-severe developmental delays and deformities in roughly 45% of the embryos. In fact, approx. 75% of the NCD-2-injected limbs displayed some type of developmental delay. For comparison, some examples of the gross deformities that we observed in the NCD-2-injected limbs are depicted in Fig. 6; we did not observe any gross deformities in the rat IgG-injected limbs, and only saw a few examples of moderate-to-severe developmental delays in this group (Table 1).

![Figure 3](image-url)
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Fig. 4. Time and dose-dependent inhibition of chondrogenesis in micromass cultures of TC cells in vitro by NCD-2. Recovered limb mesenchymal cells dissociated by TC treatment and replated back into micromass cultures (see Materials and methods) were treated with either control rat IgG antibodies (Rat IgG) or NCD-2 antibodies at various concentrations and for various lengths of time. NCD-2 treatment for the first 24 hours of culture (NCD-2 (0-24 h)) inhibited chondrogenesis in a dose-dependent manner, as demonstrated by the number of Alcian blue stained nodules after 72 hours in culture. Antibody treatment for only 15 hours after the first 9 hours (NCD-2 (9-24 h)) yielded nearly the same results as the 0-24 hours treatment, i.e. a dose-dependent inhibition of chondrogenesis. However, when NCD-2 was added to the culture medium only during the second day of incubation (NCD-2 (24-46 h)), chondrogenesis was inhibited to a less degree than the other NCD-2 treatment groups and in a non-dose-dependent manner. Control rat IgG antibodies (Rat IgG) were present in the culture for the first 24 hours of incubation. Values represent the mean ± s.d. of 4 cultures. * Denotes statistical significance (P<0.05) when compared to the matched Rat IgG-treated cultures.

DISCUSSION

In this study, we have tested the hypothesis that condensation of embryonic limb mesenchyme is mediated by Ca\textsuperscript{2+}-dependent cell adhesion molecules, or cadherins, and that this process is required for chondrogenesis. The results clearly demonstrate that N-cadherin is present and expressed in the limb mesenchyme in a development-specific manner both in vivo and in vitro, consistent with a role in mediating mesenchymal aggregation and condensation. We have also shown that functional N-cadherin is necessary for limb mesenchymal cells to undergo cellular condensation and progress through chondrogenesis in vitro and in vivo. The ability of NCD-2 to inhibit chondrogenesis in vitro is maximal during the latter half of the first 24 hours of micromass culture, which corresponds to the time period in which mesenchymal condensation takes place (San Antonio and Tuan, 1986) and when exogenous Ca\textsuperscript{2+} has its greatest stimulatory effect (San Antonio and Tuan, 1986; Tuan, 1991). NCD-2 also inhibits chondrogenesis in vivo when injected into the limb bud, causing a range of effects, from moderate developmental delays to severe deformities.

Our immunohistochemical results (Fig. 1) clearly demonstrate the pericellular N-cadherin staining to be localized to condensing regions of the limb mesenchyme. After condensation, the limb mesenchymal cells of the core region begin to down-regulate N-cadherin expression and eventually become negative, while the cells of the peripheral or cortical regions of the limb bud begin to express high levels of N-cadherin protein. Thus, in the limb bud in situ, the mesenchymal cells of the central region condense and undergo chondrogenesis, whereas the cells at the periphery and progress zone of the limb do not chondrify. We propose that only cells that express cadherins, either in situ or in vitro, will become aggregation and condensation competent and will interact with other cells that express like cadherins. After cells begin to differentiate into chondrocytes and synthesize and secrete copious amounts of cartilage extracellular matrix molecules, cell-cell contacts among the chondrocytes may be terminated as these cells become matrix encased. Overall, the general pattern of immunostaining observed in situ is also seen in micromass cultures in vitro (Fig. 1), in which condensing aggregates of mesenchymal cells appear to express the highest levels of N-cadherin.

Our cell aggregation studies strongly suggest that N-cadherin is present and functional in the chick embryonic limb mesenchyme, as TC dissociated cells, but not TE dissociated cells, displayed an aggregation profile that was Ca\textsuperscript{2+}-dependent (Fig. 1). The extent and time course of aggregation observed here are reminiscent of the data obtained by Takeichi (1977), who first described the existence of cadherins. Of particular interest was the temporal profile of NCD-2 action in blocking N-cadherin-mediated cell adhesion in suspension cultures, and chondrogenesis in micromass cultures. Initially, we observed that the length of the recovery period of the limb mesenchymal cells after initial isolation from the chick embryos was critical; both the Ca\textsuperscript{2+}-dependent aggregation of the limb mesenchymal cells, and the NCD-2 inhibition of the cellular aggregation, were optimal if the cells were allowed to recover for a period between 17-20 hours after initial isolation. Interestingly, this time period roughly corresponds to the time interval when exogenous Ca\textsuperscript{2+} is seen to stimulate chondrogenesis (San Antonio and Tuan, 1986; Tuan, 1991) and when N-cadherin is maximally expressed in micromass cultures of freshly dissociated limb mesenchyme. Taken together with the observation that cellular aggregates formed during the first 24 hours of micromass culture subsequently differentiate into cartilaginous nodules (Ahrens et al., 1977; San Antonio and Tuan, 1986; Evans and Tuan, 1988), it is reasonable to speculate that Ca\textsuperscript{2+} stimulates chondrogenesis by promoting cellular aggregation and condensation via cadherin-mediated interactions.

The second time-dependent event occurred as the cells used in the suspension cultures were resuspended and placed in culture. If the cells were first resuspended in physiologic buffer and then placed in the cultures with exogenous Ca\textsuperscript{2+} and NCD-2, they exhibited an aggregation profile that was inhibited to only a slight degree (data not shown), as compared to control antibody-treated cultures. However, if the cells were first independently resuspended in the respective antibody and control
Fig. 5. Effects of NCD-2 injection into chick limb buds in vivo. Limb buds from stages 22-24 shellless chick embryos were injected with either control rat IgG or NCD-2 antibodies, cultured for an additional 2 days, and then subjected to whole-mount Alcian blue staining as described in Materials and methods. (A-C) Site of injection visualized with Nile Blue sulfate. (A) Uninjected embryo; (B) embryo with an injected hindlimb (arrow); (C) embryo with injected fore- and hindlimb (arrows). Embryos were photographed within 1 hour of injection. (D-K) Examples of the effects of antibody injections on the morphology of primitive hindlimb (D-G) or forelimb (H-K). In D-K, the injected limb is on the right side of the photograph and the contralateral, uninjected control limb is on the left side, and digits 1, 2, 3, and 4 are as indicated; F, fibula; fi, fibulare; R, radius; T, tibia; U, ulna. (D,H) No observable effect, as compared to the control limbs. (E,J) Slight-to-moderate developmental delay. The injected limb in E showed delayed digit maturation as compared to the control limb (arrows). In I, the control limb contained a distinct phalange (arrow), which was absent from the injected limb (arrow). (F,J) Moderate-to-severe developmental delay. The injected limbs in F and J displayed a marked reduction in total digit formation as compared to the control limbs. While the control limbs contained distinct digits (arrows), there was a marked loss of skeletal formation in the injected limbs (brackets). (G,K) Gross developmental deformity. While digit formation was readily apparent in the control limbs in G and K (arrows), there was a gross loss of pattern formation and skeletal elements in the injected limbs (brackets).
solutions used in the experiments (either Ca\(^{2+}\) and NCD-2, Ca\(^{2+}\) and rat IgG, or Ca\(^{2+}\)-free buffer) and then placed in suspension cultures, the NCD-2-treated cells displayed a significant reduction in the degree of aggregation as compared to control cultures. These observations imply that recovered limb mesenchymal cells are aggregation competent immediately after TC dissociation, and, unless perturbed at this point by specific N-cadherin antibodies, will initiate cell-cell interactions upon being placed in the appropriate environment.

In micromass cultures in vitro, NCD-2 perturbs chondrogenesis in a time-dependent manner. As seen in Fig. 4, adding NCD-2 after 9 hours in culture did not appear to affect the dose-dependent inhibition of chondrogenesis by NCD-2, as compared to the 0-24 hours NCD-2 group. However, adding NCD-2 after 24 hours failed to achieve the same level of inhibition, nor the dose-dependent response. Taken together, these data imply that N-cadherin mediated cell-cell interactions do not take place in micromass culture until the latter half of the first 24 hour time period, which coincidentally, corresponds to the period of active cellular condensation in vitro. This observation makes intuitive sense since the cells, while plated at a high density into micromass cultures, may not initiate optimal cell-cell interactions immediately after plating. On the other hand, after some time in culture (probably 15-20 hours), the cells are in position to interact with one another actively and condense. This would explain the response of these cells to the NCD-2 treatment at different times. The 24-46 hours treatment is still effective, but may be limited to inhibiting cellular

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**Fig. 6.** Examples of severe limb developmental deformities resulting from injection of NCD-2 into chick embryonic limb buds in vivo. (A,C,E) Contralateral, uninjected control limbs. (B,D,F) corresponding NCD-2 injected limbs. All limbs injected and stained as described in Materials and methods. (A,B) Hindlimbs from an embryo injected at stage 24. The injected limb (B) showed normal digit formation, but was completely missing a fibula (arrow), which was present in the control (A) (arrow). (C,D) Hindlimbs from an embryo injected at stage 24. The distinct digit formation seen in C (arrow) was completely obliterated in D (bracket). Other skeletal elements were also greatly perturbed, e.g. the fibula (arrow in D). (E,F) Hindlimbs from an embryo injected at stage 24. Once again, the distinct digit formation seen in the control limb (arrow) was completely lost in the injected limb (bracket), despite the continued formation of the surrounding connective tissue elements. F, fibula; fi, fibulare; T, tibia.
recruitment into the already formed cell aggregates, whereas the earlier treatments directly perturb the initiation of cell-cell interactions, thus reducing the number of cellular aggregates formed.

It is important to compare the developmental time course of the TC isolated cells and the mesenchymal cells freshly isolated from the limb buds. Both groups of cells appear to initiate cell-cell interactions and condense at roughly the same time after plating, i.e. 15-20 hours, although the TC cells possess functional cadherins on their cell surface, whereas the freshly dissociated limb cells do not. An explanation is provided by the antibody inhibition data. The TC cells, while able to aggregate immediately upon being placed into culture, require additional environmental conditions to initiate functional cell-cell contacts. For these cells to condense, they perhaps need to orient or align themselves in the correct configuration or position, possibly through matrix-mediated mechanisms, such as matrix-driven translocation (Newman et al., 1985, 1987). In comparison, the newly dissociated limb cells must accomplish two tasks prior to active cellular condensation: (1) synthesize and express functional cadherins on their cell surface, and (2) like the TC cells, move into the correct orientation to initiate cell-cell interactions. The temporal profile suggests that these cells can synthesize and express cadherins while simultaneously aligning themselves to undergo condensation. The TC cells will still have an advantage in terms of their chondrogenic potential over the freshly isolated mesenchymal cells, but both cell types are still linked by the spatio-temporal developmental sequence of events.

While the in vitro system provides much insight into the possible sequence of events leading to chondrogenesis in the limb, the important issue of pattern formation of the developing cartilage is more adequately addressed by the in vivo whole limb injections. In one example, the NCD-2 injected limb developed without an entire fibula, but with apparently normal digits (Fig. 7B). In another severe example, the distal skeletal elements (digits) failed to form, while the overall shape and structure of the distal limb (connective tissue elements) developed and remained intact (Fig. 7F). Common to both of these limbs is that interference with N-cadherin mediated cell-cell interactions by NCD-2 successfully perturbed the development and/or growth of the cartilaginous/skeletal elements. Taken together with the cell aggregation data and temporal profile of NCD-2 inhibition on micromass cultures, we speculate that NCD-2 exerts its major effects during the condensation phase of chondrogenesis, and possibly during subsequent recruitment of mesenchymal cells into growing cartilaginous aggregates. Because the embryonic limb is dependent on the proper development of its connective tissue elements (cartilage, bone, muscle, etc.) for correct morphology and pattern formation, inhibiting chondrogenesis and therefore osteogenesis will most likely perturb overall limb formation. However, a second level of pattern formation must be addressed, i.e. pattern formation of the individual cartilaginous elements. In this study, even the extreme cases only have either missing or severely delayed cartilaginous elements with NCD-2 treatment; new or altered cartilaginous structures are not seen. Thus, NCD-2 can interfere with early events crucial for chondrocyte differentiation, but not the pattern formation of these individual elements. NCD-2 antibodies therefore act to cause a developmental delay until they are cleared (capping, endocytosis, etc.) and chondrogenesis can proceed, or the cartilaginous structure is abolished if the effective antibodies concentration remains high enough to block the mesenchymal cells from proceeding through chondrogenic differentiation. However, overall limb pattern formation may be altered simply due to gross defects in individual connective tissue elements. Although no two injections are identical in terms of tissue depth and exact location, the fact that NCD-2 injection often results in dramatic cases of chondrogenic inhibition and profound skeletal abnormalities suggests that N-cadherin is an important regulator of limb morphogenesis and pattern formation in the developing avian limb.

Once cellular interactions through cell adhesion molecules (i.e. N-cadherin and N-CAM) are initiated, what takes place to confer terminal chondrogenic differentiation? A fundamental question concerning cell adhesion and its role in morphogenesis is whether cell adhesion molecules mediate any cell regulatory mechanisms other than adhesion itself, and how this is accomplished. The classical cadherins are known to be associated with catenins (see Introduction), which mediate the attachment of the cadherins to the cytoskeleton. It is possible that extracellular binding of cadherins mechanically transduces signal(s) into the cell via the catenins and the cytoskeletal network to effect some type of regulatory mechanism(s), possibly the induction of gap junctional communication. In fact, connexin 43-mediated gap junctional communication in mouse epidermal cells has been shown to be regulated by E-cadherin in a characteristic Ca$$^{2+}$$-dependent manner (Jongen et al., 1991). A similar mechanism may be operating in the limb during chondrogenesis. Indeed, Coelho and Kosher (1991) have recently demonstrated the existence of an anterior-posterior gradient of gap junctional communication in the developing avian limb bud. Other signaling mechanisms, such as G protein-dependent L- and N-type Ca$$^{2+}$$ channels, which are regulated via N-CAM and N-cadherin actions, may also play a role (Doherty et al.,1991). In conclusion, perhaps N-cadherin, along with other adhesion molecules, mediates the condensation phase of chondrogenesis, and sets up the optimal environment in which the limb mesenchymal cells may terminally differentiate into chondrocytes. Whether N-cadherin acts in a passive or active role in regulating events subsequent to the initiation of cellular condensation remains to be elucidated.

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N-cadherin and chondrogenesis


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