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The expression of NG2 proteoglycan in the developing rat limb

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Summary

NG2 is a chondroitin sulfate proteoglycan previously found to be expressed by glial progenitor cells of the O2A lineage. We have examined the expression of NG2 in the developing rat limb by immunohistochemistry and northern blot analysis. Staining of embryonic day 14 (E14) rat limb bud sections with polyclonal and monoclonal anti-NG2 antibodies reveals reactivity in the precartilaginous mesenchymal condensation. The staining intensity increases with the differentiation of chondrocytes until E16. NG2 staining is not detected in the mature hypertrophic chondrocytes of E17 and postnatal day 3 (P3) limbs even after treatment of the sections with hyaluronidase or collagenase. Immunoprecipitations with anti-NG2 antibody using ^{125}I-labeled limb cells in culture showed a 400 to $800\times10^3 M_r$ proteoglycan species with a core protein size of $300\times10^3 M_r$, comparable to NG2 from O2A cells and neural cell lines. Northern blot analysis reveals the expression of an 8.9 kb mRNA in E16 limbs and at a lower level in P1 cartilage. The northern blot analyses also show that NG2 is distinct from the large aggregating proteoglycan of the cartilage. Our results indicate that in the developing limb cartilage, as in the differentiating oligodendrocytes, NG2 is present on immature cells in the process of differentiating, but its expression is down-regulated as terminal differentiation of chondrocytes takes place.

Key words: proteoglycan, cartilage, limb, development, type VI collagen.

Introduction

NG2 is a large chondroitin sulfate proteoglycan with a core protein of $300\times10^3 M_r$. Biochemical and immunocytochemical data suggest that the molecule is closely associated with the cell surface (Stallcup *et al.* 1983). NG2 was originally identified on rat neural cell lines with characteristics of both neuronal and glial cells (Wilson *et al.* 1981). NG2 is found in the rat optic nerve (Stallcup and Beasley, 1987) and cerebellum (Levine and Stallcup, 1987) on O2A glial progenitor cells which have the potential to differentiate *in vitro* into oligodendrocytes or type 2 astrocytes (Raff *et al.* 1983, 1984). NG2 is detected on newly formed oligodendrocytes in culture but disappears as these cells differentiate fully (Stallcup and Beasley, 1987). *In vivo* NG2 has been found on stellate, process-bearing glial cells in the rat cerebellum, but not on cells with morphological characteristics of mature oligodendrocytes (Levine and Card, 1987). These findings suggest that the expression of NG2 is turned off before the terminal differentiation of the progenitors into oligodendrocytes takes place.

We have now extended the study of NG2 distribution to extraneural tissues. To identify NG2 in various tissues, sections of developing rat embryos were screened with antibodies against the core protein of NG2. NG2 was found to be expressed in a wide variety of tissues besides the central nervous system, mostly in those of mesenchymal origin, such as the developing cartilage, skeletal muscles, smooth muscles, and heart. In the present study, we focus on the changes of NG2 expression in the developing cartilage of the limbs because the morphogenetic events of limb chondrogenesis have been well documented. Our immunohistochemical and northern blot analyses indicate that NG2 is expressed in the early stages of limb chondrogenesis but is down-regulated after the chondrocytes mature into hyaline cartilage.

Materials and methods

Cell lines

The B49 cell line was derived from an ethyl nitrosourea-induced rat brain tumor (Schubert *et al.* 1974). L14 is a cell line derived by continuous subculturing of cells obtained from primary cultures of embryonic day 14 rat limb buds. Both cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Tissue Culture Biologicals) and maintained at 37°C in the presence of 5% CO_2.

Antibodies

The derivation and specificity of polyclonal anti-NG2 and monoclonal anti-NG2 antibodies have been described (Wilson *et al.* 1981; Stallcup *et al.* 1983; Levine and Card, 1987;

Stallcup *et al.* 1990). Rabbit antibody against human type VI collagen (Hessle and Engvall, 1984) was a gift from Dr E. Engvall (La Jolla Cancer Research Foundation). The derivation of a monoclonal antibody against D1.1 ganglioside has been described (Levine *et al.* 1984). Monoclonal antibody 1C6 was obtained from Developmental Studies Hybridoma Bank.

Immunohistochemistry

Limb buds from developing Sprague–Dawley rats at embryonic day 14 (E14) to 17 (E17), postnatal day 1 (P1) and 3 (P3) were fixed by immersion with 2% paraformaldehyde in phosphate buffer, pH 7.4. The tissues were postfixed in 2% paraformaldehyde containing 30% sucrose overnight at 4°C and embedded in OCT compound (Tissue Tek). 10 μm sections were cut and mounted on gelatin- or poly L-lysine-coated slides. For indirect immunofluorescence labeling, the sections were preincubated in potassium phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100 and 2% normal goat serum (KPBS-TX-NGS) for one hour at room temperature and then incubated overnight at 4°C with primary antibodies. After four washes in KPBS-TX-NGS, the sections were incubated for 2 h at room temperature with either FITC-labeled goat anti-mouse IgG (1:50, Tago) or TRITC-labeled goat anti-rabbit IgG (1:50, Tago). For double immunofluorescence labeling, the two primary antibodies and subsequently the two secondary antibodies were applied simultaneously to the sections. After two washes in KPBS-TX-NGS, the slides were briefly rinsed in distilled water and mounted in 90% glycerol solution in phosphate buffer (pH 8.6) containing 0.25% DABCO (diazo-benzo-cyclo-octane, Sigma).

Rabbit anti-rat NG2 antibody was used at 1:1000 dilution. A mixture of six different monoclonal anti-NG2 antibodies, each recognizing a different epitope on NG2, was used at 1:100 dilution of each of the ascites fluid. Tissue culture supernatants of anti-NG2 hybridoma clones, RN143 and RN109 (Stallcup *et al.* 1990) were used at 1:3 dilution. Rabbit anti-human type VI collagen was used at 1:100 dilution. Tissue culture supernatant of D1.1 hybridoma was used at 1:3 dilution. All the dilutions were made in KPBS-TX-NGS.

To expose any epitopes that might be masked by the extracellular matrix, some sections were treated with 0.1 to 1 mg ml^{-1} of bovine testicular hyaluronidase (Calbiochem) in phosphate-buffered saline (PBS), pH 7.4, at room temperature for 30 min or 0.5 mg ml^{-1} of type VII collagenase (Sigma) in PBS at 37°C for 30 min and rinsed three times in PBS prior to incubation with the primary antibodies.

Immunoprecipitations

Cultured cells were surface labeled with ^{125}I using the lactoperoxidase method (Hubbard and Cohn, 1972). Detergent extracts were made using 1% Nonidet P-40 (NP-40), and insoluble material was removed by centrifugation. Rabbit anti-NG2 antibody was added to the extracts and allowed to incubate at room temperature for 1 h. Antigen–antibody complexes were precipitated with protein A–Sepharose beads and washed three times in phosphate buffer containing 0.1% NP-40. Chondroitinase digestion was carried out by treating the immunoprecipitates with 0.02 unit of chondroitinase ABC (Miles) at room temperature overnight. The complexes were dissolved by boiling the pellets in electrophoresis sample buffer containing 3% SDS and 5% 2-mercaptoethanol and electrophoresed on 3–20% polyacrylamide gradient gels (Laemmli, 1970). The gels were dried and exposed for autoradiography with Kodak XAR-5 film.

Western blot analysis

Aggrecan was prepared from Swarm rat chondrosarcoma (a gift from Dr Paul Goetinck, La Jolla Cancer Research Foundation, CA). Extracts were made with 4 M guanidine–HCl and subjected to isopycnic CsCl density gradient centrifugation as described by Hascall and Kimura (1982). The bottom one-fifth of the dissociation gradient (G1) was collected. One-half of this fraction was reduced and alkylated by treating the extracts with 0.1 M 2-mercaptoethanol at room temperature for 30 min followed by incubation in 0.11 M iodoacetamide at room temperature for 30 min. Crude B49 cell extracts were obtained by extracting the cells with 1% NP-40. A portion of the samples was digested with chondroitinase ABC overnight at room temperature at a final concentration of 1.0 unit ml^{-1}. The digested and undigested samples were boiled in a buffer containing 3% SDS and 5% 2-mercaptoethanol, electrophoresed through 3–20% acrylamide gradient gels and blotted onto Immobilon membranes (Millipore, MA) in a buffer containing 10 mM CAPS, pH 11.0, and 10% methanol. The filters were blocked overnight at room temperature in 5% BSA and 10% newborn calf serum and subsequently incubated with either a mixture of six anti-NG2 monoclonal antibodies (each at 1:150 dilution) or the ascites fluid of hybridoma 1C6 (Caterson *et al.* 1986; 1:500 dilution) for 2 h at room temperature. 1C6 antibody recognizes a defined epitope within the hyaluronic acid-binding region of aggrecan. After four washes, the filters were incubated in horseradish peroxidase-labeled goat anti-mouse immunoglobulins (1:2000 dilution, Bio-Rad) for 2 h at room temperature. The filters were developed using diaminobenzidine and hydrogen peroxide.

Northern blot analysis

Total RNA fractions were prepared from cell lines by the guanidinium isothiocyanate method (Chirgwin *et al.* 1979). Poly (A)$^+$ RNA was extracted from developing rat limb and brain tissues by lysing the tissues in the presence of 2% SDS and 200 mg ml^{-1} of proteinase K (Boehringer-Mannheim), directly followed by oligo (dT)-cellulose (Collaborative Research) affinity chromatography (Badley *et al.* 1988). 10 μg of total RNA or 2.5 μg of poly (A)$^+$ RNA were fractionated in 1% agarose gels containing 2.2 M formaldehyde according to the method of Thomas (1980) and blotted onto nitrocellulose filters. The filters were hybridized with ^{32}P-labeled cDNA probes. For detecting NG2 mRNA, a 600 bp cDNA fragment representing the 5′ coding region of NG2 was used. Details on the isolation of NG2 cDNA clones will be presented elsewhere. A 1.0 kb cDNA fragment from the coding region of the large aggregating proteoglycan of the rat cartilage, aggrecan (Doege *et al.* 1987), which was a gift from Dr K. Doege (Shriners Hospital for Crippled Children, Oregon), was also used. Preparation of labeled probes was done by the random priming method of Feinberg and Vogelstein (1983) or by nick translation (Rigby *et al.* 1977). The probe had an activity of 5×10^6 cts min^{-1} ml^{-1}. The filters were washed to a final stringency of 60°C in a buffer containing 0.2×SSC and 0.1% SDS and exposed for autoradiography with Kodak XAR-5 film.

Results

(1) Immunohistochemistry

The temporal and spatial changes in NG2 expression in the developing rat limb from E14 to P3 were examined by indirect immunofluorescence. By double immuno-

fluorescence labeling, the distribution of NG2 was compared with that of type VI collagen and D1.1 ganglioside. Type VI collagen has been found to interact with the core protein of NG2 and may be an extracellular ligand of NG2 (Stallcup *et al.* 1990). D1.1 is a cell surface ganglioside which has previously been

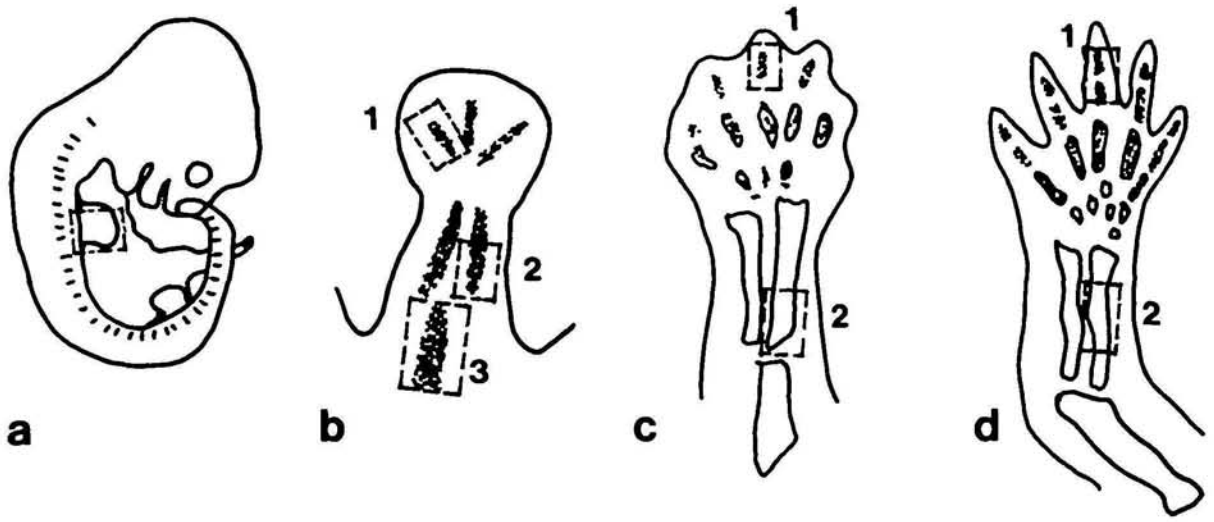


Fig. 1. A diagram showing different stages of the developing rat limb. (a) E14 embryo. Box represents field shown in Fig. 2. (b) E15 forelimb. Box 1 represents field shown in Fig. 3, a–d; box 2, Fig. 3, e–h box 3, Fig. 3, i–l. (c) E16 forelimb. Box 1 represents field shown in Fig. 4; box 2, Fig. 5. (d) E17 hindlimb. Box 1 represents field shown in Fig. 6; box 2, Fig. 7.

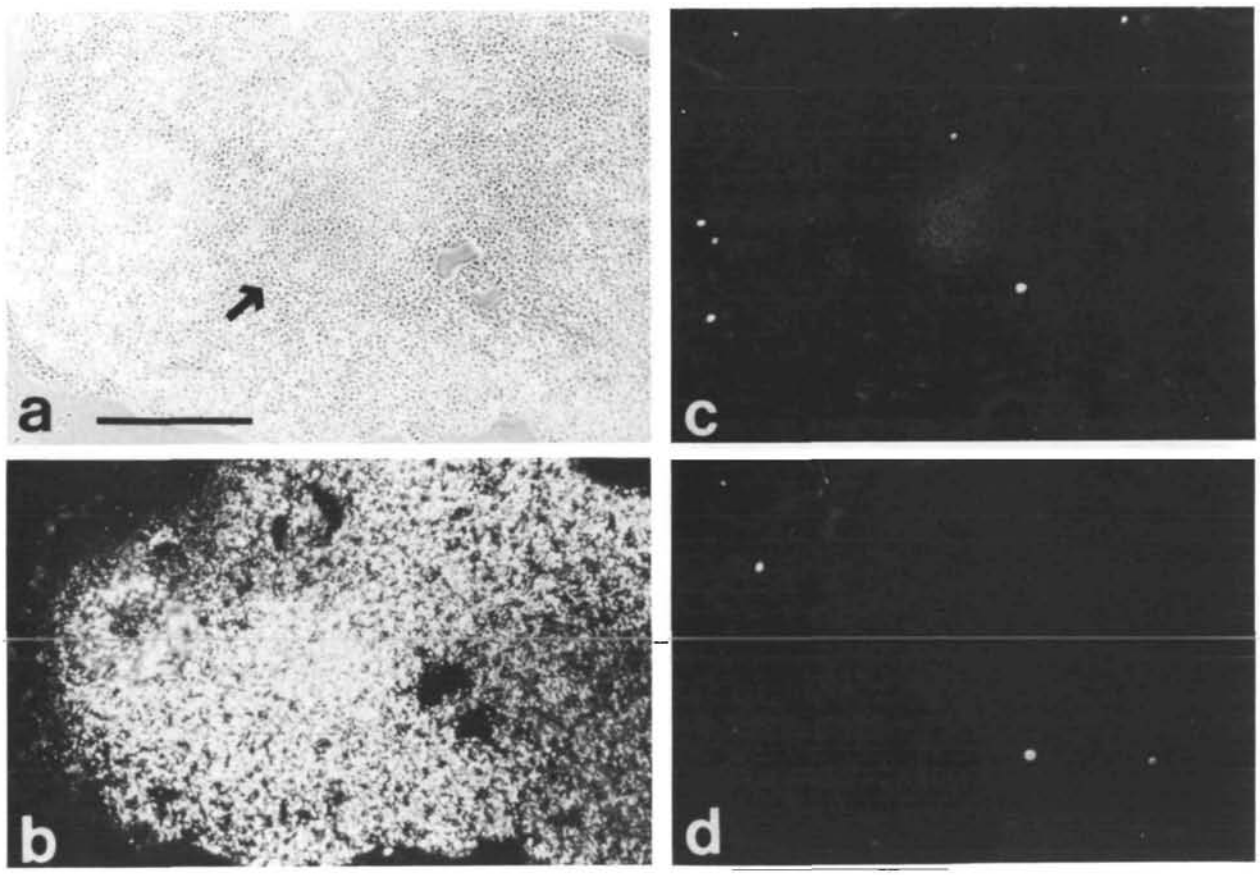


Fig. 2. Indirect immunohistochemical labeling of an E14 limb bud. (a) Phase contrast. Note area of condensed cells in the center (arrow). (b) Same section stained with anti-D1.1 monoclonal antibody. (c and d) Double labeling of an adjacent section with a mixture of six anti-NG2 monoclonal antibodies (c) and rabbit anti-type VI collagen antibody (d). Scale bar in a=500 μm.

shown to be a marker for proliferating neuroepithelial cells in the developing central nervous system (Levine *et al.* 1984).

(A) E14 limbs

The limb bud of an E14 rat embryo appears as a small protrusion from the trunk (Fig. 1a). A longitudinal section through the limb bud shows a confined central area of condensed mesenchymal cells, surrounded by loose connective tissue cells and a surface epithelium (Fig. 2a). At this stage NG2 immunoreactivity is restricted to the region of mesenchymal condensation (Fig. 2c). The staining is characterized by ring-like fluorescence, which we interpret to be cell surface staining rather than extracellular matrix staining. No significant staining above background is seen with anti-type VI collagen antibody (Fig. 2d). D1.1 immunoreactivity is diffusely detected both on the condensed cells and the surrounding loose mesenchymal cells but is absent from the epithelium (Fig. 2b).

(B) E15 limbs

In E15 embryos, the limb bud appears as a short shaft with a flat, elongated, paddle-shaped structure at the end (Fig. 1b). A longitudinal section through an E15 limb bud reveals a proximodistal gradient of cartilage differentiation. In the distal region (Fig. 3, a–d), the cartilage anlage for the digits is seen as a prechondro-

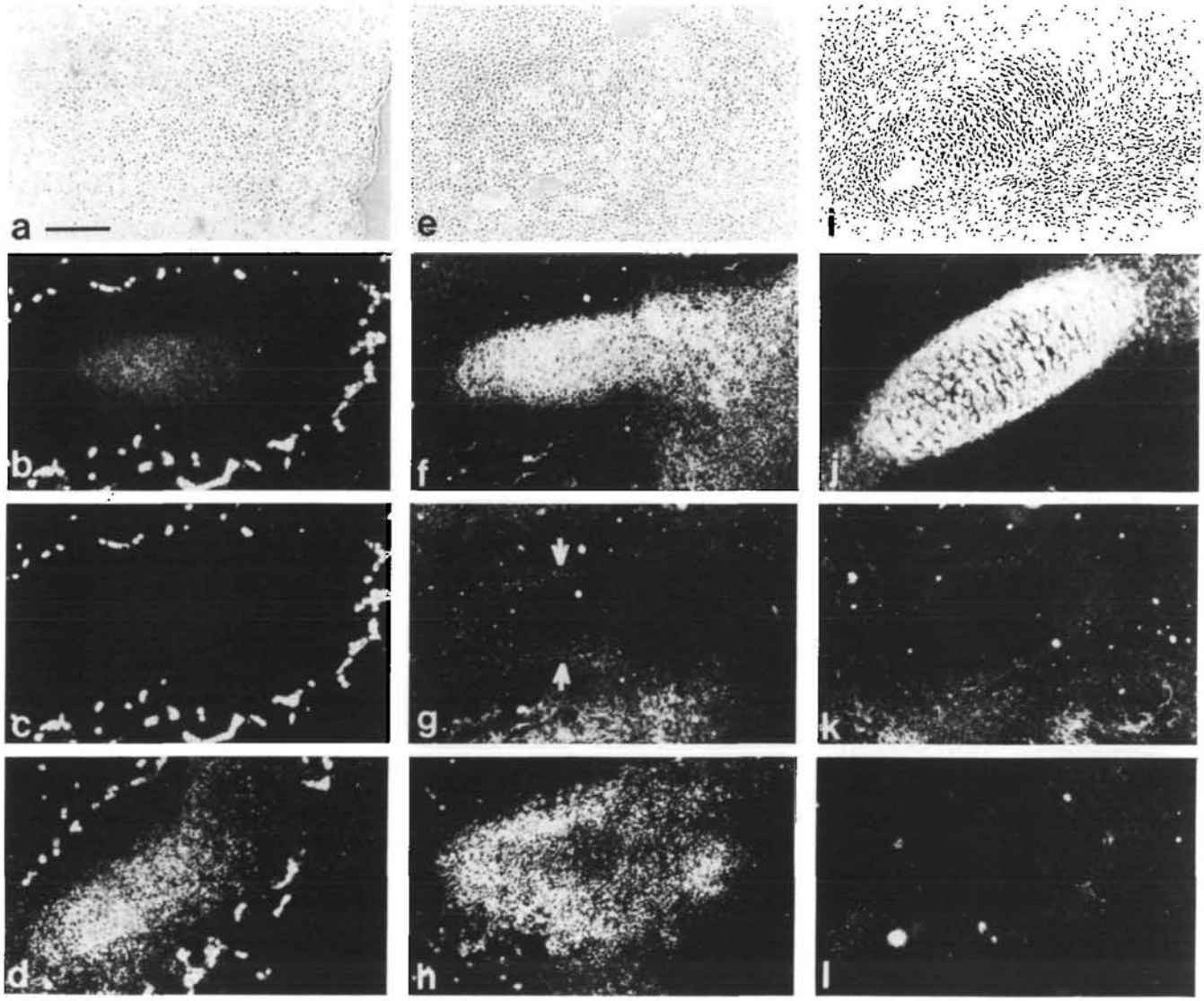


Fig. 3. Immunofluorescence labeling of the E15 digit (a–d), radius (e–h), and humerus (i–l). (a, e, i) Phase contrast. (b and c, f and g, j and k) Double labeled with anti-NG2 monoclonal antibody mix (b, f, j) and rabbit anti-type VI antibody (c,g,k). (d, h, l) Adjacent sections stained with monoclonal anti-D1.1 antibody. The proximodistal gradient of cartilage differentiation is apparent. NG2 labeling is stronger in the more proximal regions of the limb. Type VI collagen is faintly detected in the distal core (c), present at the periphery of the radius blastema (g, arrows) but is absent from the more mature chondrogenic tissue in the humerus (k). D1.1 labeling is concentrated in the chondrogenic tissue, with decreasing levels in the more mature tissue. The prominent fluorescence in the blood vessels surrounding the digit anlage (b,c,d) is nonspecific staining caused by blood cells. Scale bar in a=200 μm.

genic mesenchymal cell condensation, surrounded by more loosely arranged connective tissue cells separated from the core by prominent blood vessels (Fig. 3a). NG2 immunoreactivity is present on the condensed mesenchymal cells but not in the surrounding mesenchyme (Fig. 3b). Double immunofluorescence labeling of the same section with anti-type VI collagen antibody displays a weak staining of the condensed mesenchymal cells (Fig. 3c). Staining of an adjacent section for D1.1 also shows immunoreactive cells in the chondrogenic region, but labeled cells are spread over a somewhat wider area (Fig. 3d).

In the radius anlage, the condensed mesenchymal cells are larger and oval-shaped (Fig. 3e). NG2 is strongly expressed on these differentiating chondrocytes (Fig. 3f). Type VI collagen staining is seen only on the cells at the periphery of the developing cartilage blastema (Fig. 3g, arrows). By double immunofluorescence labeling, these marginal cells are shown to be immunoreactive for both NG2 and type VI collagen. D1.1 is more widely distributed with less staining in the central core (Fig. 3h).

More proximally in the humerus anlage, the cells are larger and show some resemblance to mature hypertrophic chondrocytes (Fig. 3i). These chondrocytes are intensely labeled with anti-NG2 antibody. NG2 immunoreactivity is absent from the surrounding non-chondrogenic tissue (Fig. 3j). The small, round cells of the elbow and shoulder joints at each end of the humerus also express NG2. In the chondrogenic tissue of the humerus, type VI collagen staining is less prominent than that seen at the periphery of the radius, though staining is present in the surrounding loose connective tissue (Fig. 3k). D1.1 staining is absent from the central core of the humerus anlage (Fig. 3l).

(C) E16 limbs

The limb bud of an E16 embryo is more elongated than the E15 limb bud and has a wider distal end (Fig. 1c). The proximodistal gradient noted at E15 is still apparent in the E16 limb. The cartilage anlage for the digits can be seen as a condensed mesenchymal core consisting of cells which are slightly larger than the surrounding mesenchymal cells (Fig. 4a). NG2 immunoreactivity can be detected in these cells by the typical ring-like cell surface fluorescence (Fig. 4c). Anti-type VI collagen antibody gives weak staining of a small population of cells at the periphery of the cartilage anlage (Fig. 4d, arrows). D1.1 also labels the central core, but staining is detected over a wider area than NG2-positive cells (Fig. 4b). The cytoarchitecture and the staining pattern revealed by the antibodies to the three different antigens in the E16 digit are comparable to those of the E15 radius anlage.

A section through the E16 elbow joint shows large, differentiating chondrocytes in the radius and smaller,

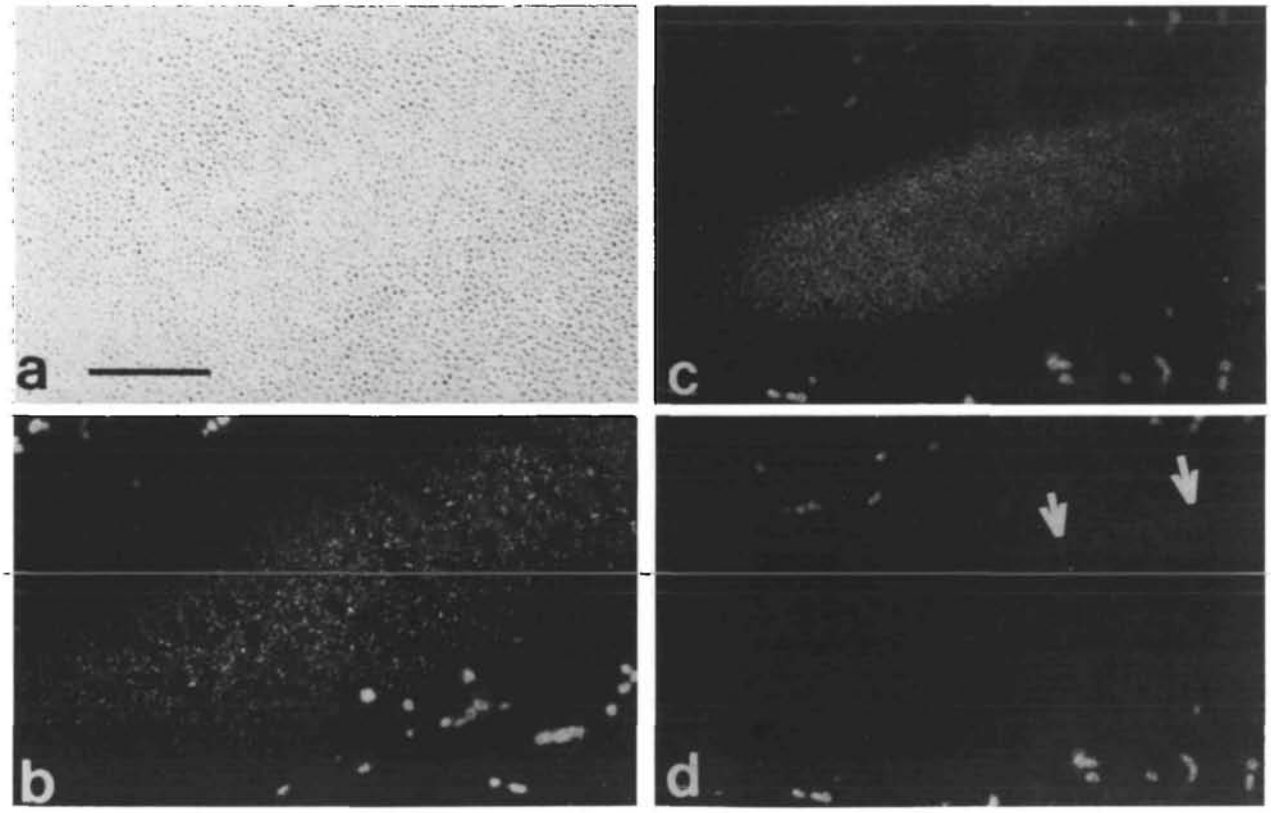


Fig. 4. Immunofluorescence labeling of the E16 digit anlage. (a) Phase constrast. (b) Same section stained with monoclonal anti-D1.1 antibody. (c and d) Double staining with anti-NG2 monoclonal mix (c) and rabbit anti-type VI collagen (d). Note ring-like cell surface fluorescence of NG2 in c. There is weak type VI collagen staining on the cells at the periphery of the blastema (d, arrows). Scale bar in a=200 μm.

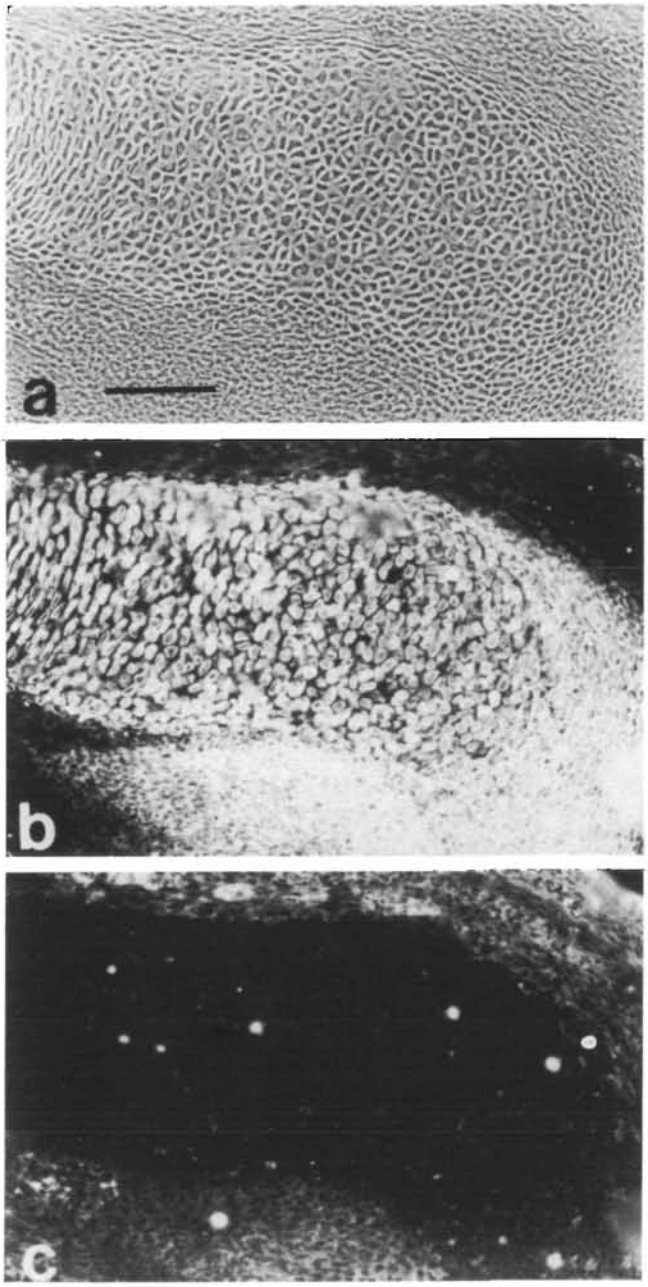


Fig. 5. Immunofluorescence labeling of the proximal end of the radius and the elbow joint of the E16 limb. a: Phase contrast. (b and c) Double labeled with anti-NG2 monoclonal antibody mix (b) and rabbit anti-type VI collagen (c). NG2 is detected on large chondrocytes of the radius and small, round cells of the joint, whereas type VI collagen is only present on the latter and in the connective tissue. Scale bar=200 μm.

round cells of the elbow joint (Fig. 5a). Anti-NG2 antibody stains both of these structures intensely (Fig. 5b). Anti-type VI collagen antibody does not label the central core of the radius but stains some cells in the joint and the surrounding connective tissue (Fig. 5c). The structure and immunoreactivity of the E16 radius are comparable to those of the E15 humerus.

(D) E17 limbs

The E17 limb has the appearance of a mature limb with an elongated shaft and separated digits (Fig. 1d). A longitudinal section through the limb shows that the digits have differentiated into individual phalanges connected by the developing joint tissue. The cartilage rudiment of each phalanx consists of large cells resembling mature hypertrophic chondrocytes (Fig. 6a). These cells are weakly stained with anti-NG2 antibody (Fig. 6c). Stronger NG2 immunoreactivity is detected on the small round cells of the interphalangeal joint (Fig. 6c), which are also stained, but less intensely, with anti-type VI collagen (Fig. 6d) and D1.1 (Fig. 6b) antibodies.

More proximal regions of the E17 limb shows characteristics of mature hyaline cartilage. Fig. 7 shows a section of the tibia, in which the cartilage consists of mature hypertrophic chondrocytes, surrounded by a few layers of cells that constitute the perichondrium (Fig. 7a). Anti-NG2 antibodies do not stain the hypertrophic chondrocytes and label the perichondrium very weakly (Fig. 7c). Anti-type VI collagen and D1.1 antibodies stain some cells of the perichondrium and the surrounding connective tissue but not the chondrocytes (Fig. 7c and 7b).

No NG2 labeling is seen in any part of the cartilage tissue of the P1 or P3 limb. Treatment of sections with bovine testicular hyaluronidase or collagenase prior to immunofluorescence labeling fails to reveal NG2- or type VI collagen-immunoreactive cells. Staining of sections with polyclonal or monoclonal anti-NG2 antibodies all give comparable results.

(2) Immunoprecipitations

In order to confirm the identity of the antigen recognized by the polyclonal and monoclonal anti-NG2 antibodies in the developing limb sections, ^{125}I-labeled cell extracts from B49 neural cells and L14 limb cells were subjected to immunoprecipitation with polyclonal anti-NG2 antibody. When the immunoprecipitates were run on a 3–20% acrylamide gradient gel, NG2 was detected as a broad high molecular weight component ranging from 400×10^3 M_r to 800×10^3 M_r in both cell lines (Fig. 8, lanes 1 and 3). Treatment of the immunoprecipitates with chondroitinase ABC results in an increase in the intensity of the 300×10^3 M_r band and a loss of the high molecular weight broad component in both cell lines (lanes 2 and 4), suggesting that NG2 core protein in the brain and the limb are identical. As discussed by Stallcup *et al.* (1990), the prominent band at 140×10^3 M_r in the case of B49 cells is composed of light chains of type VI collagen which is coprecipitated as a complex with NG2. The 200×10^3 M_r band seen after chondroitinase treatment is a proteolytic fragment of the 300×10^3 M_r core protein. Prior to chondroitinase treatment this fragment contains chondroitin sulfate chains and migrates in the lower molecular weight portion of the NG2 smear. Some of this fragment is also seen in the case of L14 cells.

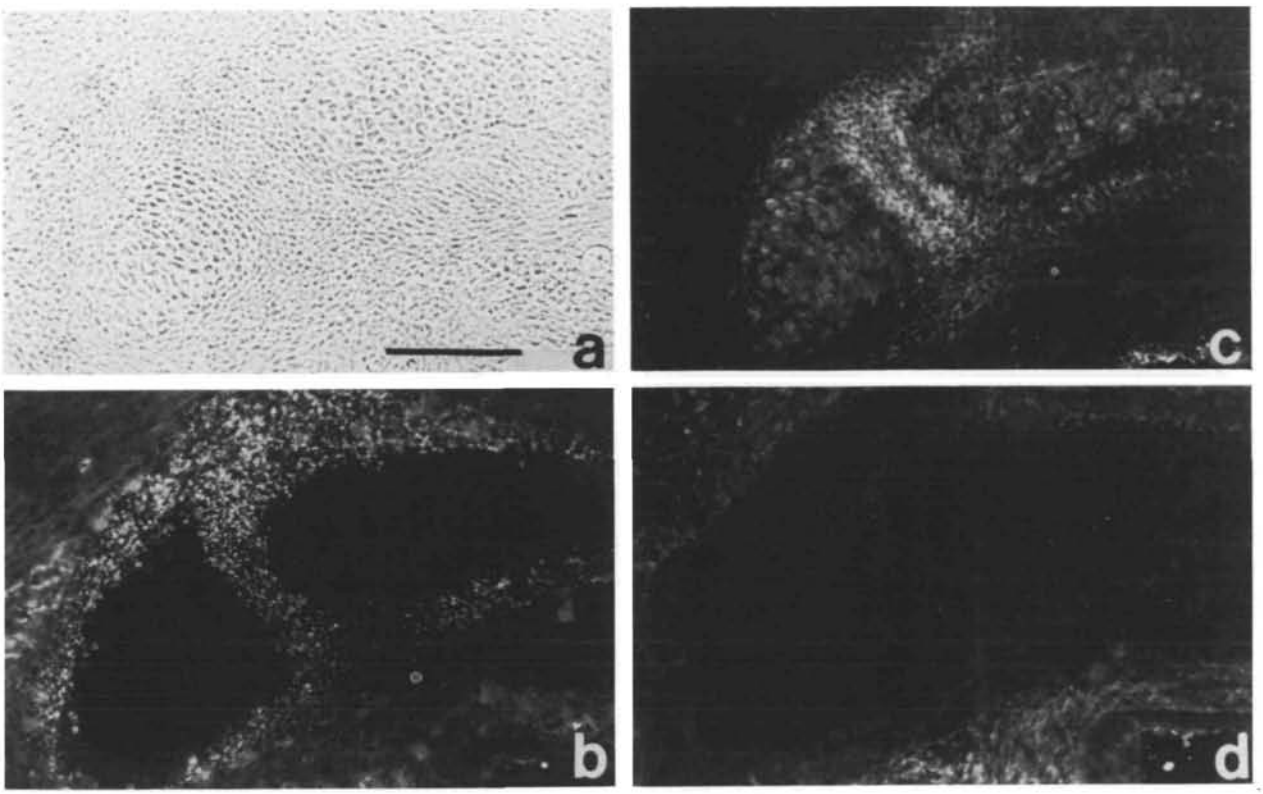


Fig. 6. Immunofluorescence labeling of an E17 digit. (a) Phase contrast. (b) Same section stained with monoclonal anti-D1.1 antibody. (c and d) Double labeling of an adjacent section with anti-NG2 monoclonal antibody mix (c) and rabbit anti-type VI collagen (d). NG2 immunoreactivity of the chondrocytes has become weaker. D1.1 and type VI collagen overlap with NG2 in the joint between phalanges. Scale bar in a=200 μm.

(3) Western blot analysis

Western blots were performed to test the specificity of anti-NG2 monoclonal antibodies. Extracts from Swarm rat chondrosarcoma containing aggrecan and extracts from B49 cells containing NG2 were run on 3–20% acrylamide gradient gels and transferred to Immobilon membranes. The filters were incubated with a mixture of six different monoclonal antibodies against NG2 or monoclonal antibody 1C6 which recognizes the hyaluronic acid-binding region of aggrecan. As shown in Fig. 9, 1C6 reacts with chondroitinase-treated G_1 fractions of chondrosarcoma extracts (panel A, lanes 1 and 3), revealing two high molecular weight bands larger than 200×10^3 M_r (arrows) and one smaller band of 100×10^3 M_r (arrowhead). The two large species correspond to core I and core II derived from the large aggregating proteoglycan as described by Kimura *et al.* (1981) and Stevens *et al.* (1984). The 100×10^3 M_r band may be a proteolytic product of the large core proteins that contains the hyaluronic acid-binding region. The high molecular weight proteoglycan species cannot be detected in undigested samples either because it is too large to enter the gel or because of inefficient transfer. 1C6 antibody does not recognize B49 cell extracts (Fig. 9A, lanes 5 and 6). The mixture of anti-NG2 monoclonal antibodies recognizes the 300×10^3 M_r core protein of NG2 in chondroitinase-digested B49 cell extracts (Fig. 9B, lane 5) and the proteoglycan smear in the undigested extract (Fig. 9B, lane 6), but does not react with Swarm chondrosarcoma extracts (Fig. 9B, lanes 1–4). These results indicate that the anti-NG2 monoclonal antibodies do not cross-react with aggrecan.

(4) Northern blot analysis

To determine whether the changes in the expression of NG2 observed by immunohistochemistry occur at the mRNA level, we performed northern blot assays using poly $(A)^+$ RNA fractions from different stages of the developing rat limb. Hybridization of a northern blot with an NG2 cDNA probe reveals an 8.9 kb mRNA in the L14 cell line and E16 limbs (Fig. 10a, lanes 3 and 1) but not in P1 cartilage (Fig. 10a, lane 2). In some experiments, an 8.9 kb mRNA can be detected in early postnatal limb cartilage tissues, but the intensity of the signal is lower than that of the E16 limbs (data not shown). A similar 8.9 kb mRNA is also detected in P7 and P16 brain tissues and in the B49 neural cell line (Fig. 10a, lanes 4, 5 and 6). When the same filter is hybridized with a cDNA probe encoding the rat large aggregating proteoglycan (aggrecan), an 8.9 kb mRNA is detected weakly in E16 limbs and much more strongly in P1 cartilage and L14 cells (Fig. 10b, lanes 1, 2 and 3). No aggrecan mRNA is detected in P7 and P16 brain

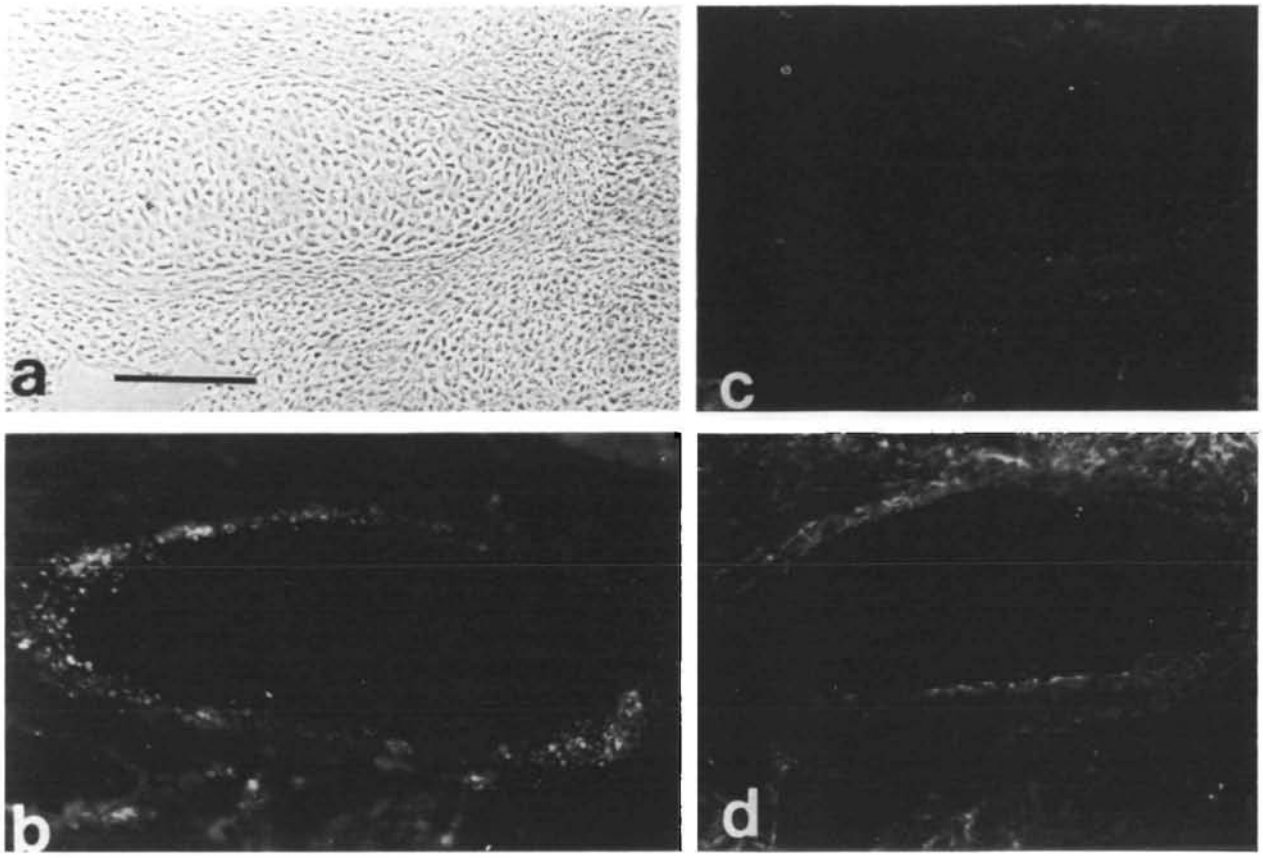


Fig. 7. Immunofluorescence labeling of an E17 tibia. (a) Phase contrast. (b) Same section labeled with anti-D1.1 antibody. (c and d) Double labeled with anti-NG2 monoclonal antibody mix (c) and rabbit anti-type VI collagen (d). NG2 is undetectable in the hypertrophic chondrocytes. NG2, D1.1 and type VI collagen are present in the perichondrium. Scale bar in a=200 μm.

tissues or in the B49 cell line (Fig. 10b, lanes 4, 5 and 6). These results show that NG2 mRNA is distinct from aggrecan mRNA, and that, unlike aggrecan mRNA, NG2 mRNA is more abundantly expressed in immature cartilage than in differentiated cartilage.

Discussion

In the present study, we have demonstrated the presence of NG2 in the developing rat limb and have shown its expression to be developmentally regulated. The following observations suggest that the NG2 antigen detected in the limb is the same molecule as that found in the brain. First, immunoreactivity is observed in the limb tissues not only with a rabbit antiserum against NG2, but also with several different monoclonal antibodies which recognize different epitopes on NG2 of neural origin. Second, immunoprecipitations of limb cell (L14) extracts with anti-NG2 antiserum reveal a proteoglycan species of 400 to 800×10^3 M_r with a core protein size of 300×10^3 M_r, similar to NG2 found in neural cell lines and O2A progenitor cells. The mobility of the intact NG2 proteoglycan from L14 cells is slightly greater than that from B49 cells, suggesting differences in the chondroitin sulfate chains of the two core proteins. Third, northern blots of RNA from both limb and brain tissues reveal mRNA species of 8.9 kb which hybridize with an NG2 cDNA probe, indicating that NG2 is translated from the same mRNA in the two tissues. Therefore, we conclude that NG2 proteoglycan is not specific to the nervous system but is found in other developing systems such as differentiating cartilage.

The results of western blot analyses demonstrate that the monoclonal anti-NG2 antibodies used in this study do not cross-react with aggrecan. This indicates that the immunofluorescence in the chondrogenic tissues observed in the present study is a result of specific reaction of anti-NG2 antibodies to NG2 in these tissues and not due to cross-reactivity of the antibodies to aggrecan.

With the exception of some immunoreactivity in endothelial and smooth muscle cells of arteries, most of the NG2 staining in the E14 and E15 limbs seems to be confined to the chondrogenic tissue. Beginning at E16, and especially after E17, prominent labeling can be seen in the skeletal muscle cells (not shown).

Anti-NG2 antibodies reveal a unique temporal pattern of NG2 expression in the developing cartilage. NG2 is first detected in the E14 limb bud at the site of mesenchymal condensation. With the progression of cartilage differentiation, the intensity of NG2 staining

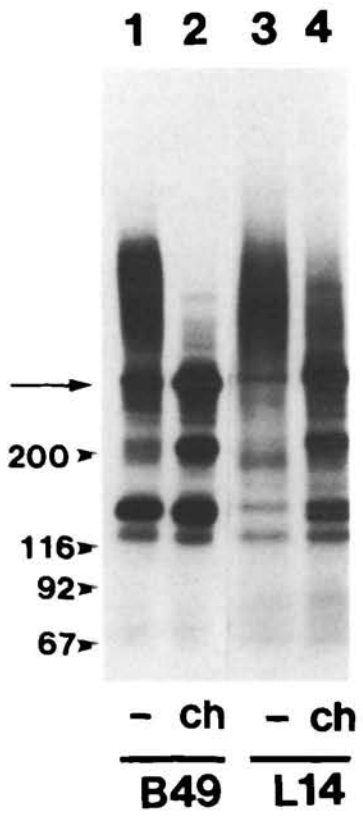


Fig. 8. Immunoprecipitations of ^{125}I-labeled B49 and L14 cells with anti-NG2 polyclonal antibody. (Lane 1) The immunoprecipitate from B49 cells. Control (-). (Lane 2) Immunoprecipitate from B49 cells treated with chondroitinase ABC (ch). (Lane 3) Immunoprecipitate from L14 cells. Control (-). (Lane 4) Immunoprecipitate from L14 cells treated with chondroitinase ABC (ch). NG2 proteoglycan appears as a broad component of $400–800\times10^3$ M_r, with a core protein of 300×10^3 M_r (arrow) in both B49 and L14 cells.

increases. Strongest immunoreactivity is found on the differentiating chondrocytes of E15 and E16 limbs just before they undergo terminal differentiation into mature, hypertrophic chondrocytes. NG2 can no longer be detected in fully differentiated hyaline cartilage. The distribution of NG2 during the early stages of chondrogenesis is similar to that reported for aggrecan (Mallein-Gerin *et al.* 1988). However, unlike NG2, aggrecan is present at high levels in the mature cartilage.

To determine whether NG2 is indeed absent from the differentiated cartilage or whether the antigen is masked in the complex extracellular matrix, sections were treated with hyaluronidase and collagenase. Although hyaluronidase digestion slightly increased the staining intensity of the large differentiating chondrocytes of E17 digits, neither hyaluronidase nor collagenase treatment resulted in visualization of NG2 in the more mature hypertrophic chondrocytes of E17 or P3 cartilage.

Northern blot analysis of RNA extracted from different stages of the developing limb reveals the presence of NG2 mRNA in both E16 and P1 limbs, with higher levels of expression at E16. Hybridization of the same filter with an aggrecan cDNA probe shows that aggrecan mRNA is much more abundant in P1 cartilage than in E16 limbs. Furthermore, no aggrecan mRNA is detected in the brain tissues which contain NG2 mRNA. These results indicate that NG2 and aggrecan are distinct gene products having different patterns of expression during development of the limb cartilage. This point is further supported by our findings that there is no similarity between the complete cDNA sequence of NG2 (Nishiyama *et al.* submitted) and that of aggrecan (Doege *et al.* 1987). The presence of low levels of NG2 mRNA in the mature cartilage tissue may be due to contamination of the cartilage preparation by muscle tissue, which is positive for NG2 at this stage of development. Alternatively, NG2 mRNA may be present in the mature cartilage, but the NG2 core may not be detectable by immunofluorescence due to translational regulation or post-translational modifications.

Several different species of proteoglycans have been shown to increase as the limb mesenchymal cells differentiate into chondrocytes (Kimata *et al.* 1986; Shinomura *et al.* 1984; Noro *et al.* 1983; Mallein-Gerin *et al.* 1988; Matsui *et al.* 1989; Sommarin and Heinegard, 1986). Of these, aggrecan is known to be the major constituent of the differentiating cartilage. Most of the other molecules differ from NG2 in their estimated size and tissue distribution. PG-M, which is a large, hyaluronic acid-binding chondroitin sulfate proteoglycan isolated from chick limb buds (Kimata *et al.* 1986), seems to share some properties with NG2. These include a high ratio of protein to glycosaminoglycans, reflecting the presence of few, large chondroitin sulfate chains. However, PG-M seems to be more widely distributed in the limb mesenchyme than NG2. A more detailed comparison of the proteoglycans must await further structural characterization of these two molecules.

The onset of limb chondrogenesis is manifested by condensation of mesenchymal cells (Fell and Canti, 1934) accompanied by a transient increase in the synthesis of type I collagen (Dessau *et al.* 1980) and fibronectin (Dessau *et al.* 1980; Tomasek *et al.* 1982; Kulyk *et al.* 1989) and a decrease in hyaluronic acid (Singley and Solursh, 1981). Fibronectin has been implicated in the intercellular adhesion that occurs during condensation (Tomasek *et al.* 1982; Kulyk *et al.* 1989). We have found that NG2 and fibronectin are colocalized only in the mesenchymal condensation during the initial phase of chondrogenesis (data not shown). Whether NG2 is involved in cell adhesion remains unknown. In the present study, we have demonstrated that immunoreactivity for a cell surface ganglioside D1.1 is initially diffusely distributed in the limb bud. D1.1 reactivity later becomes confined to the chondrogenic tissue and is lost from the mature hypertrophic cartilage, showing a temporal pattern of expression similar to fibronectin. D1.1 has been localized in dividing neuroectodermal cells of the developing central nervous system (Levine *et al.* 1984).

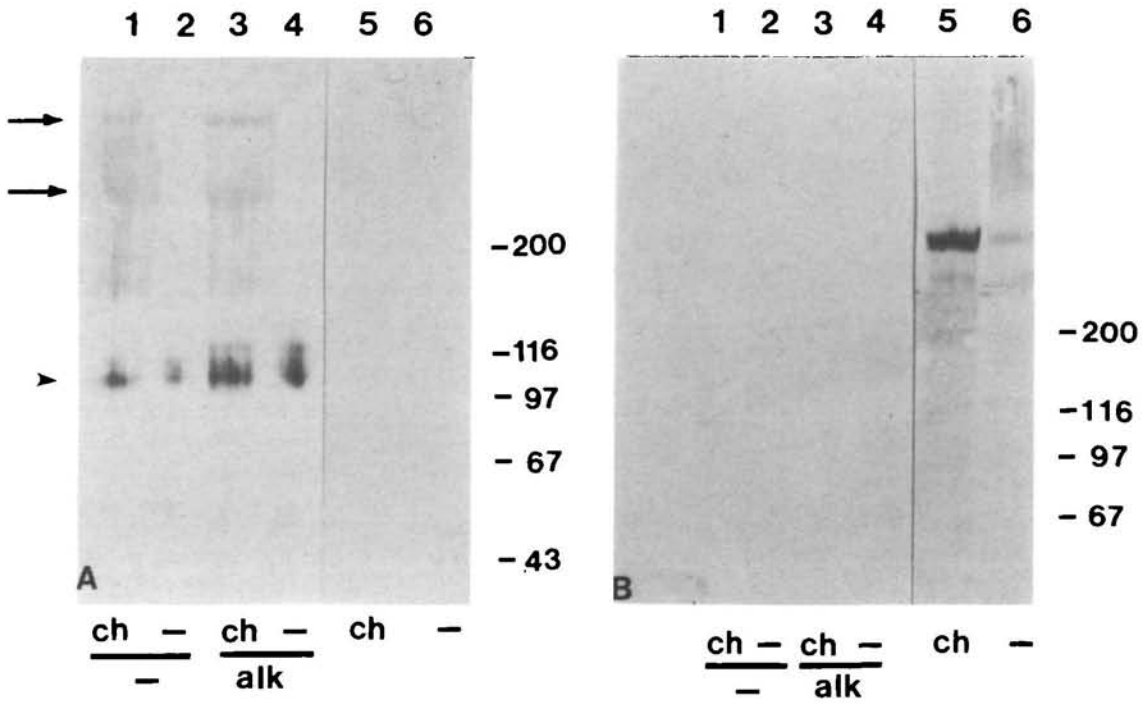
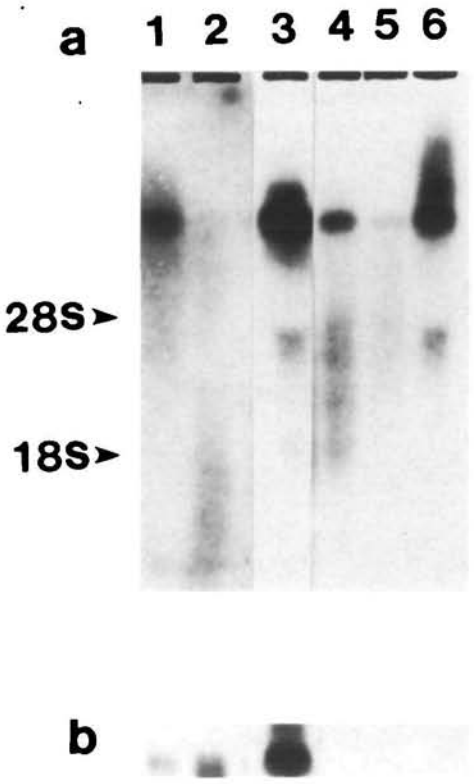


Fig. 9. Western blot analysis of B49 cell and Swarm rat chondrosarcoma extracts with anti-NG2 monoclonal antibodies and monoclonal anti-hyaluronic acid-binding region antibody 1C6. (A) Incubated with 1C6 antibody (ascites, 1:500). (B) Incubated with a mixture of six monoclonal anti-NG2 antibodies (ascites, 1:150 each). Lanes 1–4. G_1 fraction of density gradient of Swarm rat chondrosarcoma extract. Lanes 3 and 4. Reduced and alkylated after CsCl density gradient centrifugation (alk). Lanes 5 and 6. 1% NP40 extract of B49 cells. Lanes 1, 3, and 5. Treated with chondroitinase ABC (ch). Lanes 2, 4, and 6. Untreated (-). Anti-NG2 monoclonal antibodies do not recognize cores I and II (arrows) or the fragment (arrowhead) of the cartilage proteoglycan in chondrosarcoma extracts. Conversely 1C6 antibody does not react with NG2 in B49 cell extract.



It is of interest that D1.1 is also present on the undifferentiated, proliferating cells of the early limb bud. It has been postulated that D1.1 might play a modulatory role in the binding of fibronectin to its receptor in the central nervous system (Stallcup, 1988; Stallcup *et al.* 1989). D1.1 might play a similar role in modifying cell adhesion in the developing cartilage tissue during the mesenchymal cell condensation stage.

We were interested in comparing the distribution of NG2 with that of type VI collagen since it has been shown that type VI collagen can associate with NG2 through a protein–protein interaction (Stallcup *et al.* 1990). There have been a few reports on the distribution of type VI collagen in fetal and adult cartilage tissues with somewhat conflicting results (Keene *et al.* 1988;

Fig. 10. Northern blot analysis of limb and brain tissues. (a) Hybridized with ^{32}P-labeled 600 bp cDNA probe from the coding region of NG2. (b) Hybridized with ^{32}P-labeled 1000 bp cDNA from the coding region of rat aggrecan. (Lane 1) 2.5 μg of poly $(A)^+$ RNA from E16 limb. (Lane 2) 2.5 μg of poly $(A)^+$ RNA from P1 limb cartilage. (Lane 3) 15 μg of total RNA from L14 cells. (Lane 4) 5.0 μg of poly $(A)^+$ RNA from P7 cerebellum. (Lane 5) 5.0 μg of poly $(A)^+$ RNA from P16 brain. (Lane 6) 15 μg of total RNA from B49 cells. 8.9 kb NG2 mRNA is detected in E16 limbs, L14 cells, P7 and P16 brain tissues, and B49 cells. 8.9 kb aggrecan mRNA is detected in E16 limbs, P1 cartilage, and L14 cells, but not in the brain or B49 cells.

Ayad *et al.* 1984; von der Mark *et al.* 1984), but changes in the expression of type VI collagen during cartilage development have not been described in detail. Our immunohistochemical studies show that type VI collagen is first faintly detected in the condensed cells which are labeled with anti-NG2 antibodies. As the chondrocytes start to differentiate, type VI collagen disappears from the central core and becomes localized to the periphery of the cartilage blastema, marking the outer boundary for the NG2-positive cluster of cells. With the progression of chondrogenesis, type VI collagen is transiently expressed in the perichondrium and later becomes undetectable in the mature cartilage tissue. Type VI collagen staining persists in the loose connective tissue. The role of type VI collagen in chondrogenesis is presently unknown. Like type I and type II collagens, which have been shown to enhance proteoglycan synthesis in somite explants (Kosher and Church, 1975), type VI collagen might stimulate NG2 synthesis. Alternatively, by interacting with cell surface NG2, type VI collagen might regulate other events of chondrogenesis. Our sequence data obtained from NG2 cDNA clones indicate that the NG2 core is an integral membrane protein. Thus NG2 could be involved in transmitting signals from the extracellular matrix to the cytoplasm, thereby causing changes in gene expression. Of interest in this context are the striking similarities in the distribution of NG2 and retinoic acid gamma receptor (Ruberte *et al.* 1990). Further studies on extracellular ligands and intracellular signals specified by NG2 may be helpful in understanding the role of NG2 in chondrogenesis and other differentiation processes.

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