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Perlecan is a component of cartilage matrix and promotes chondrocyte attachment

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SUMMARY

Aggrecan, a chondroitin/keratan sulfate-containing proteoglycan, is a major component of cartilaginous tissues. Immunolocalization studies, using antibodies directed to perlecan, a heparan sulfate proteoglycan first detected in basement membranes, and laminin (another major component of basement membranes), indicate that perlecan and laminin are also present in the matrices of hyaline cartilage in the nasal septum, the articular surface of the bone and the growth plate of the developing bone. Consequently, we used antibodies to both aggrecan and perlecan to characterize their synthesis and secretion by primary cultures of chondrocytes derived from the rat chondrosarcoma. Chondrocytes were pulsed for 20 minutes with [^{35}S]methionine and then chased for up to six hours. The radiolabeled perlecan and aggrecan were immunoprecipitated and analyzed by SDS-PAGE. The results show that chondrocytes synthesize precursor proteins to both proteoglycans, but that only the aggrecan precursor protein is secreted as a proteoglycan. Perlecan was also secreted but with less posttranslational modifications than aggrecan. Northern blot analyses of the RNAs from immortalized rat chondrocytes indicated that the major mRNA encoding for perlecan was approximately 13 kb in length, similar in size to that expressed by other cell types, which synthesize 400 kDa core protein perlecan. Analyses of the proteoglycan fractions from the extracts of bovine articular surface indicated that perlecan in this tissue contains both chondroitin and heparan sulfate side-chains. Purified perlecan and laminin were found to promote attachment of immortalized rat chondrocytes in vitro. These studies indicated that perlecan, once thought to be a unique component of the basement membranes, is more widely distributed and is an important component of the cartilage matrix, where it may provide for cell adhesion to the matrix.

Key words: perlecan, cartilage, chondrocyte

INTRODUCTION

Hyaline cartilages are specialized connective tissues which consist of chondrocytes embedded in their abundant extracellular matrices. Hyaline cartilage matrices are mainly composed of collagen II, aggrecan (a chondroitin/keratan sulfate-containing proteoglycan), link protein, cartilage matrix proteins and hyaluronate. Three of these components, aggrecan, link protein and hyaluronate interact to form large aggregates which serve to resist compression, and thereby provide the principle function of the matrix of cartilage (Heinegård and Oldberg, 1989; Hascall et al., 1991). Aggrecan also plays an important role in the growth of cartilaginous structures. The failure to produce aggrecan in two mutants, the CMD mouse (Argraves et al., 1981) and the nanomelia chick (Kimata et al., 1981), results in shortened long bones and is lethal, presumably due to a collapsed trachea.

It is evident from the histochemical and immunohistochemical staining characteristics of the cartilage matrix that it exhibits regional specialization into pericellular, territorial and interterritorial matrices (Scott and Dorling, 1965; Stockwell and Scott, 1967). Regional differences in the cartilage matrix composition probably are functionally critical in hyaline cartilage of the growth plate. During endochondral ossification, the matrix is likely to influence the continuous behavioral and metabolic changes in the chondrocytes, which continuously undergo proliferation and differentiation.

Like cartilage matrices, which are characterized by their unique components, basement membranes form another type of specialized matrix, which is characterized by having collagen IV, laminin, entactin/nidogen and perlecan, a heparan sulfate-containing proteoglycan as its constituents (Aumailley, 1993). These gene products are unrelated to the gene products that comprise major components of cartilage matrix and thus have different functions. Basement membranes are thin sheets of extracellular matrix which serve to separate dissimilar tissues, to act as a barrier to cell migration or to provide a fluid filter. The non-collagenous components of basement membranes, laminin (Graf et al., 1987), entactin (Chakravarti et al., 1990) and perlecan (Clement et al., 1989; Hayashi et al.,

1992), are also excellent substrata for cell attachment. In contrast, the noncollagenous components of the cartilage matrix have not been associated with cell-attachment activities. In the present study, we found that some of the basement membrane components are also present in the hyaline cartilage, including the articular cartilage and the cartilage of the growth plate. These components, once thought to be restricted to the basement membranes, have a wider tissue distribution; and may have a specialized role in cell-matrix interactions during the growth and differentiation of chondrocytes.

MATERIALS AND METHODS

Preparation of cell cultures

Cells were isolated from the Swarm rat chondrosarcoma as previously described (Kimura et al., 1979). In brief, tumor was harvested at 4-6 weeks postimplantation, diced into 2 mm^3 pieces and pressed through a 1 mm^2 stainless steel sieve to further dissociate the tumor tissue. The tissue was then minced in calcium/magnesium-free Hanks' balanced salt solution (HBSS) and the cells dissociated with 0.5% trypsin (Gibco/BRL, Grand Island, NY) and 0.4% collagenase (Worthington, Freehold, NJ) at 37°C for one hour. The digest was filtered through a 70 μm nylon screen and the cells pelleted by centrifugation. The pelleted cells were washed in HBSS by centrifugation and then plated out in Dulbecco's modified Eagle's medium (DME) containing 20% fetal calf serum (FCS), at 1.0×10^6 cells/35 mm dish.

Immortalized rat chondrocytes (IRC) were grown as aggregates in suspension culture in Ham's F-12 medium (Gibco/BRL) containing 10% FCS (Horton et al., 1988). The aggregates were harvested by low speed centrifugation and the pelleted aggregates were either: (1) resuspended in fresh medium and passaged for continued suspension culture; or (2) dissociated by digestion with 0.4% collagenase in HBSS at 37°C for 20 minutes. The dissociated cells were then pelleted by low speed centrifugation and resuspended in HBSS containing 1% BSA and used in cell attachment assays.

Radiolabeling and Immunoprecipitation

Cultured chondrosarcoma cells were 'pulsed' with 500 μCi [^{35}S]methionine/ml (DuPont/NEN, Boston, MA) in methionine-free medium for 20 minutes and then 'chased' in medium lacking radiolabeled methionine for up to 6 hours as previously described (Ledbetter et al., 1985). The medium was removed and the cells solubilized in lysis buffer. Perlecan, aggrecan or laminin in the medium and the cell lysate were immunoprecipitated using Protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) containing bound IgG to either perlecan, aggrecan or laminin, respectively (Ledbetter et al., 1985). The radiolabeled immunoprecipitated proteins were separated by SDS-PAGE, the gel was embedded in Fluoro-Hance (Research Products International Corp., Mt Prospect, IL) and the dried gel exposed to X-OMAT X-ray film (Eastman Kodak Co., Rochester, NY) at −80°C.

Extraction and fractionation of articular cartilage

One to two millimeters of the articular surface of bovine bones was shaved off with a knife and extracted in a solvent containing 4 M guanidine-HCl and protease inhibitors (Oegema et al., 1975). The extract was fractionated on a column (1.5 cm × 90 cm) of Sepharose CL-4B in 4 M guanidine-HCl containing 0.05 M Tris-HCl, pH 7.0. Aliquots (50 μl) from each tube were analyzed for aggrecan and perlecan content by dot blot analysis, as described below. The tubes containing perlecan were pooled, and dialyzed against 6 M urea containing 0.15 M NaCl and 0.05 M Tris-HCl, pH 6.8, and then applied to DEAE-Sepharose in the same solvent. After collecting the pass-through fraction (glycoprotein), the column was washed with 1.15 M NaCl in the same solvent to collect the proteoglycan fraction. The glycoprotein and proteoglycan fractions were tested for the presence of perlecan by dot blot analysis as described below. Aliquots of proteoglycan fractions were also digested with heparatinase and chondroitinase ABC (Seikaguku America, Inc., Rockville, MD) and then analyzed by SDS-5% PAGE followed by the western blot technique (Kato et al., 1985; Klein et al., 1988).

Dot blot and western blot analyses

Aliquots (50 μl) of each fraction eluted from the Sepharose CL-4B column were applied to nitrocellulose membrane using a Bio-Dot (Bio-Rad) apparatus. After blocking the membrane with 5% milk powder and 1% Tween-20 in Tris-buffered saline, the membranes were incubated with rabbit anti-mouse perlecan or rabbit anti-chicken aggrecan antibodies. ECL (Amersham Life Sciences Inc., Arlington Heights, IL) reagent was then used to detect the antibody binding using the manufacturer's protocol. Briefly, after the treatment with primary antibodies, the membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit antibodies followed by ECL detection reagent, and the membranes were then exposed to X-ray films for autoradiography. The relative densities of dots on the autoradiograms were determined using a scanner and a computerized image analysis system. Similarly, ECL reagents were also employed for the detection of aggrecan and perlecan bands on western blots.

RNA isolation and northern blot

Total RNA was isolated from pelleted aggregates of IRC cells using the guanidine isothiocyanate-phenol-chloroform method (Chomczymski and Sacchi, 1987), electrophoresed on a 1% agarose gel containing formaldehyde, transferred to Gene-Screen (DuPont-NEN England Nuclear), and then crosslinked and immobilized with ultraviolet light (Stratalinker; Stratagene, La Jolla, CA). Non-overlapping cDNA clones to domains I and II, domain III, domain IV and domain V of mouse perlecan (Noonan et al., 1991) were individually used as templates to prepare ^{32}P-labeled probes using a random prime labeling kit (Pharmacia Biotechnology, Piscataway, NJ) and [^{32}P]dCTP (3000 Ci/mmol; DuPont-NEN). The Gene-Screen blot was cut into individual lanes and, after prehybridization, each lane was hybridized overnight at 42°C with one of the ^{32}P-labeled domain-specific probes for perlecan. The Gene-Screen strips were then washed at high stringency according to the protocols for Gene Screen and exposed at −80°C to X-OMAT X-ray film with an intensifying screen.

Immunostaining

Adult human nasal cartilage with the surrounding tissues, day 15 embryonic chick sternum, a tarsal joint of 21-day-old newborn mouse and rat chondrosarcoma were each frozen in OCT compound (Tissue Tek II, Miles, Inc., Elkhart, Ind.) and stored frozen at −70°C. Cryostat sections (7 μm) of the frozen tissues were transferred to gelatin-coated slides and reacted with specific antibodies (either directly or after treatment with chondroitinase) using an indirect rhodamine-conjugated antibody technique as described previously (SundarRaj et al., 1988). Chondroitinase treatment was carried out by incubating the sections on the slides with a drop (50 μl) of chondroitinase solution (2 units/μl) for 15 minutes. The slides were washed with PBS and then immunostained with specific antibodies. The stained sections were mounted in Aqua-Mount (Lerner Labs, New Haven, CN) and examined and photographed with a photomicroscope with fluorescence attachments and epifluorescence objectives. For comparing relative densities of staining, the same exposures - 1 to 5 seconds - were used to photograph each set of sections stained with each different antibody.

To describe the immunostaining pattern in the different regions of cartilage matrix, the matrix was defined into different zones according to Poole et al. (1980, 1982). The matrix zone immediately surrounding the chondrocytes at the edges of the lacunae, is referred to as the pericellular zone; the wider zone surrounding the pericellular zone is

defined as the territorial zone; and the rest of the matrix, between the territorial zones, is referred to as the interterritorial matrix.

Cell attachment assays

A 1 cm × 5 cm strip of nitrocellulose was dissolved in 6 ml of 100% methanol and 50 μl of this solution was spread on each well of a 24-well culture dish and the methanol allowed to evaporate. Each of the wells were then coated with perlecan, laminin or BSA in 0.5 ml of PBS for 24 hours at 4°C. The fluid was removed and replaced with 0.5 ml of 3% BSA in PBS for 24 hours at 4°C to block any remaining binding sites on the dish. IRC cells were biosynthetically radiolabeled overnight in suspension culture with 25 μCi of [^{3}H]thymine/10 ml medium. The IRC aggregates were harvested by centrifugation, and the cells dissociated by collagenase digestion as described above. The cells and coated plates were rinsed once in Ham's F-12 containing 0.05% BSA, the cell number determined and ≈50,000 cells added per well. After $1\frac{1}{2}$ hours of incubation at 37°C the unattached cells were removed by two rinses with PBS. The attached cells were solubilized with 0.5 ml 1% SDS and counted for radioactivity. The percentage attachment was determined as the percentage of radioactivity in 50,000 cells.

Antisera and basement membrane components

Antisera were raised in rabbits against perlecan purified from the EHS tumor (Hassell et al., 1980), against aggrecan purified from the rat chondrosarcoma (Doege et al., 1986) and from chick scleral cartilage (Rada and Matthews, 1994). Monoclonal antibody to human laminin was developed in our laboratory and polyclonal anti-laminin antisera were purchased (Calbiochem Corp., La Jolla, CA). Monoclonal antibody to human collagen IV (SundarRaj and Wilson, 1982) was used for immunostaining of chick, rat and bovine tissues. Perlecan and laminin were purified from the EHS tumor as previously described (Hassell et al., 1980; Graf et al., 1987) for use in cell attachment assays.

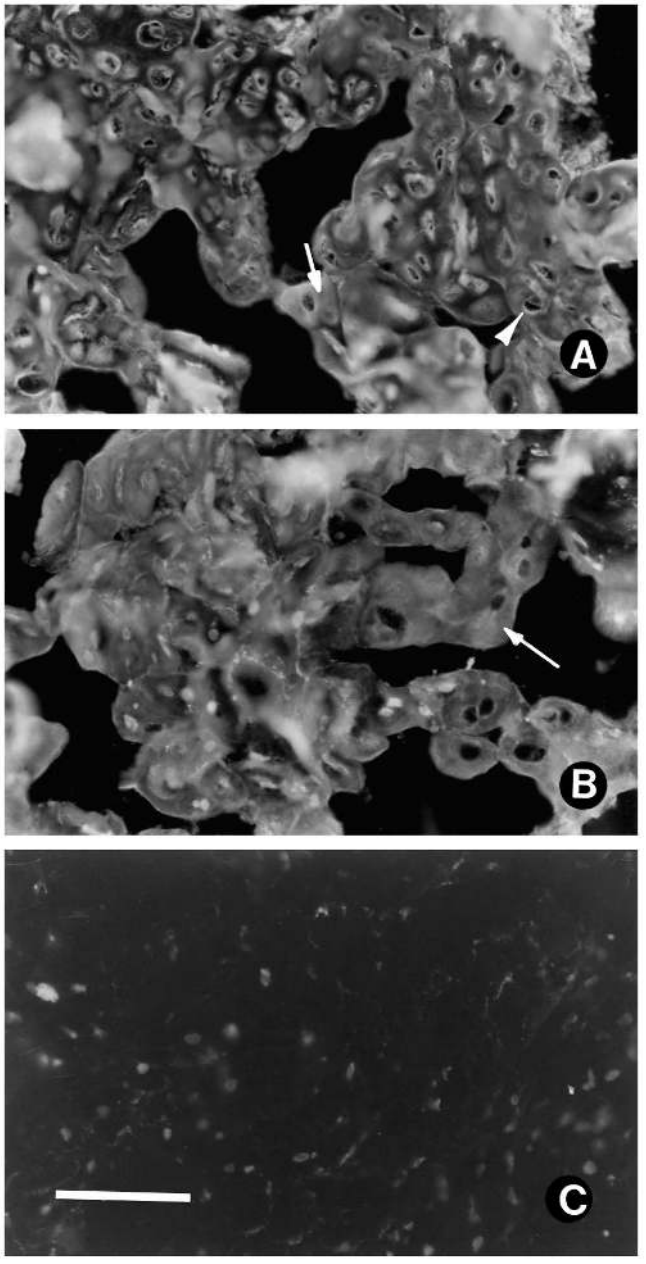


Fig. 1. Indirect immunofluorescence staining of rat chondrosarcoma. Cryostat sections of the tumor were immunoreacted with: (A) polyclonal rabbit anti-perlecan; (B) polyclonal rabbit anti-aggrecan; (C) preimmune serum followed by FITC-conjugated anti-rabbit antibody. Staining for perlecan (A) is evident in the matrix around the cells and is more intense in the pericellular region (arrowhead). Intense staining for aggrecan (B) is evident throughout the matrix surrounding the cells (arrow). Bar, 100 μm.

RESULTS

The presence and distribution of perlecan and aggrecan were analyzed on sections of rat chondrosarcoma, a transplantable tumor cell line that produces a cartilage matrix. Antisera to perlecan intensely stained not only the pericellular matrix but also, somewhat less intensely, the surrounding matrix (Fig. 1A). Antisera to aggrecan, stained both the pericellular and the surrounding matrices with similar intensities (Fig. 1B).

The rat chondrosarcoma was also used to follow the biosynthesis and cell secretion of both perlecan and aggrecan. The chondrosarcoma was dissociated into single cells, the cultured cells pulsed for 30 minutes with [^{35}S]methionine and chased

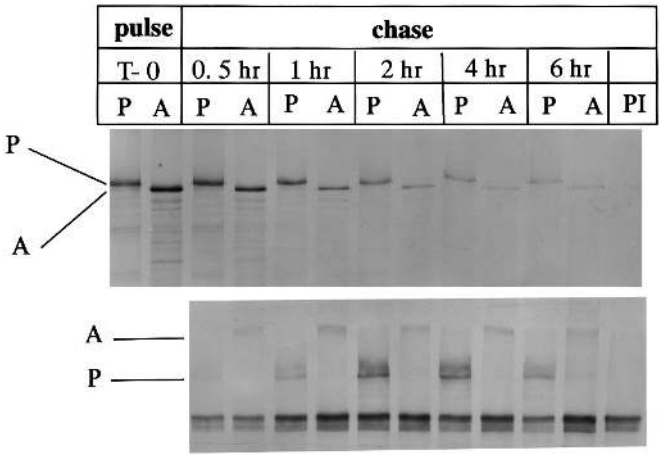


Fig. 2. Autoradiograms of the immunoprecipitated proteins from [^{35}S]methionine-labeled chondrosarcoma cells. Cells were metabolically pulse-labeled with [^{35}S]methionine and the label was chased into the medium for the indicated period of time. Labeled perlecan (P) and aggrecan (A) in the cell layer (top panel) and culture media (bottom panel) were immunoprecipitated with polyclonal antibodies to perlecan or aggrecan, separated by SDS-PAGE and visualized by autoradiography.

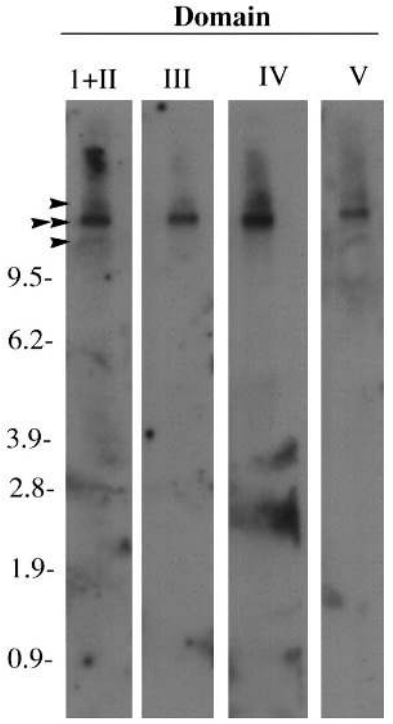


Fig. 3. Northern blot analysis of RNA from immortalized rat chondrosarcoma cells (IRC). Total RNA isolated from IRC grown in culture as aggregates were subjected to northern blot analyses using cDNAs to different domains of perlecan as the probes. The major mRNA which hybridized with each of the cDNA probes was approximately 13 kb in size (double arrowhead). A slightly larger mRNA which hybridized with all the probes (top arrowhead) and a slightly smaller mRNA (bottom arrowhead) which hybridized with domains I and II probes were also detected but were considerably lighter on the autoradiograms.

for up to 6 hours. Antisera to perlecan immunoprecipitated the previously identified 400 kDa precursor protein to perlecan (Ledbetter et al., 1985) and antisera to aggrecan the previously identified and slightly smaller precursor protein to aggrecan (Kimura et al., 1981) from the extracts of the pulsed cell layers (Fig. 2). cDNA cloning has since shown that aggrecan is derived from a 220 kDa precursor protein. Aggrecan precursor protein migrates abnormally on SDS-PAGE because of the structure of its GAG-binding domain. The concentrations of radiolabeled precursor proteins to perlecan (P) and aggrecan (A), immunoprecipitated from the cell layer, decreased during the 6 hour chase (Fig. 2, upper panel). Aggrecan immunoprecipitated from media during the chase period (Fig. 2, lower

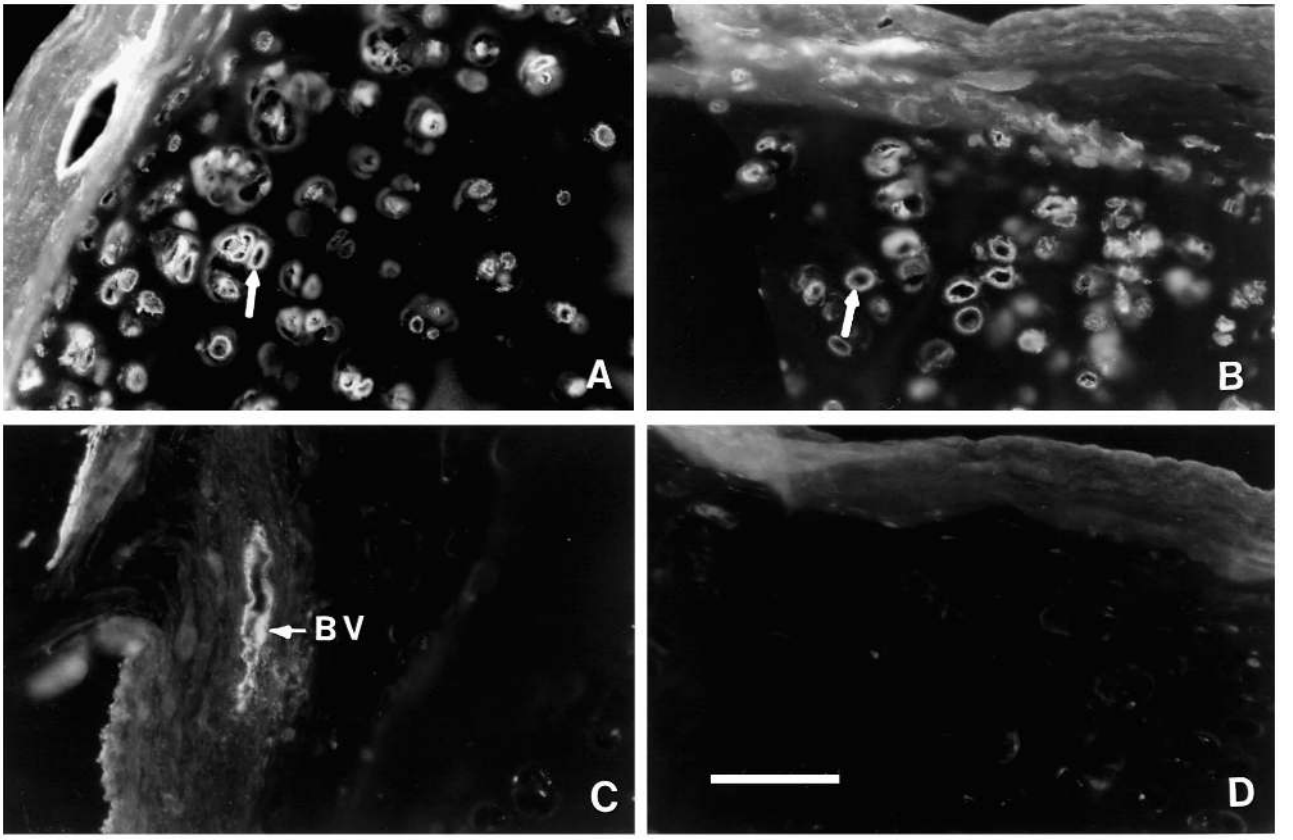


Fig. 4. Indirect immunofluorescence staining of human nasal cartilage. Cryostat sections of human nasal cartilage were immunostained with the following specific mouse monoclonal or rabbit polyclonal antibodies followed by FITC-conjugated second antibody: (A) polyclonal anti-perlecan; (B) monoclonal anti-laminin; (C) monoclonal anti-type IV collagen; and (D) preimmune rabbit serum. Staining of perlecan (A) and laminin (B) was very strong in the pericellular region (arrows), weaker in the territorial matrix and negligible in the interterritorial matrix. Staining of type IV collagen (C) was not detectable in the cartilage matrix; however, it was strong in the walls of blood vessels (BV) in the perichondrium. Bar, 100 μm.

panel) consisted of a product that just entered the running gel. This is the aggrecan core protein with GAG side-chains, which indicates conversion to a proteoglycan (Kimata et al., 1981). In contrast, antisera to perlecan immunoprecipitated a closely spaced doublet, the lower band being the same size as the precursor protein detected in the cell layer. In contrast to the results with chondrocytes, the pulse-chase experiment previously conducted with cultured EHS cells showed the secreted perlecan product to increase in size during the chase period (Ledbetter et al., 1985). This suggests that in chondrocyte cell cultures perlecan is synthesized and secreted with limited post-translational modifications.

Similar studies on immortalized rat chondrocytes (IRC) showed that they synthesized and deposited perlecan into their extracellular matrix (data not shown). Therefore, we determined the size of the perlecan message in RNA isolated from IRC cells, using cDNA clones to all five domains of perlecan (Fig. 3) as the probes. The major mRNA detected with each cDNA was ≈13 kb but all of cDNA probes also detected a slightly higher molecular mass band of lesser intensity, and cDNA to domains I and II detected a slightly lower molecular mass band of lesser intensity. The sizes of these mRNAs are comparable to the size of the mRNA in EHS cells which encodes for the 400 kDa perlecan precursor protein (Noonan et al., 1988).

We also examined native cartilage for the presence of basement membrane components. Antibodies to perlecan reacted intensely with the pericellular matrix of human nasal

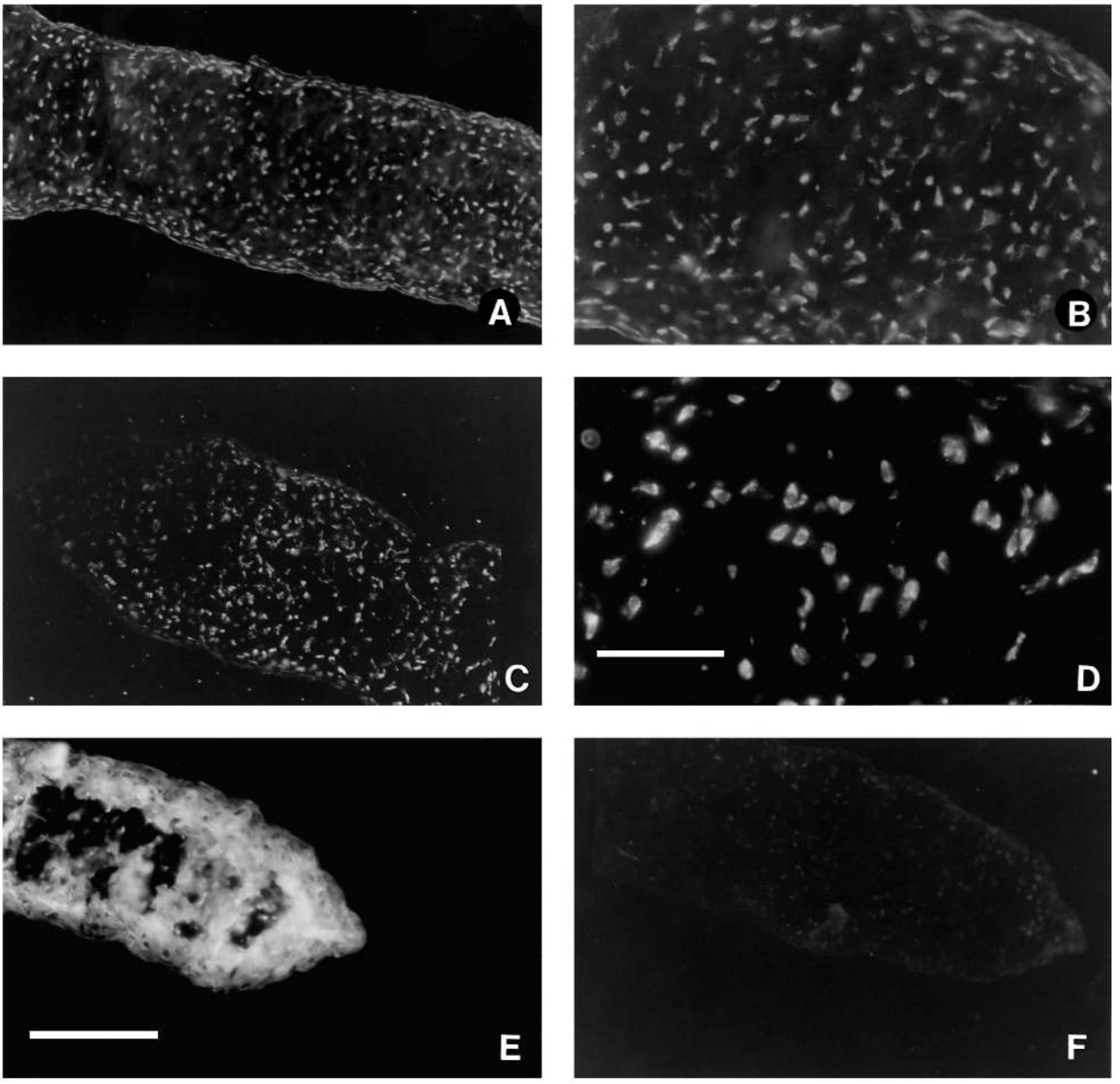
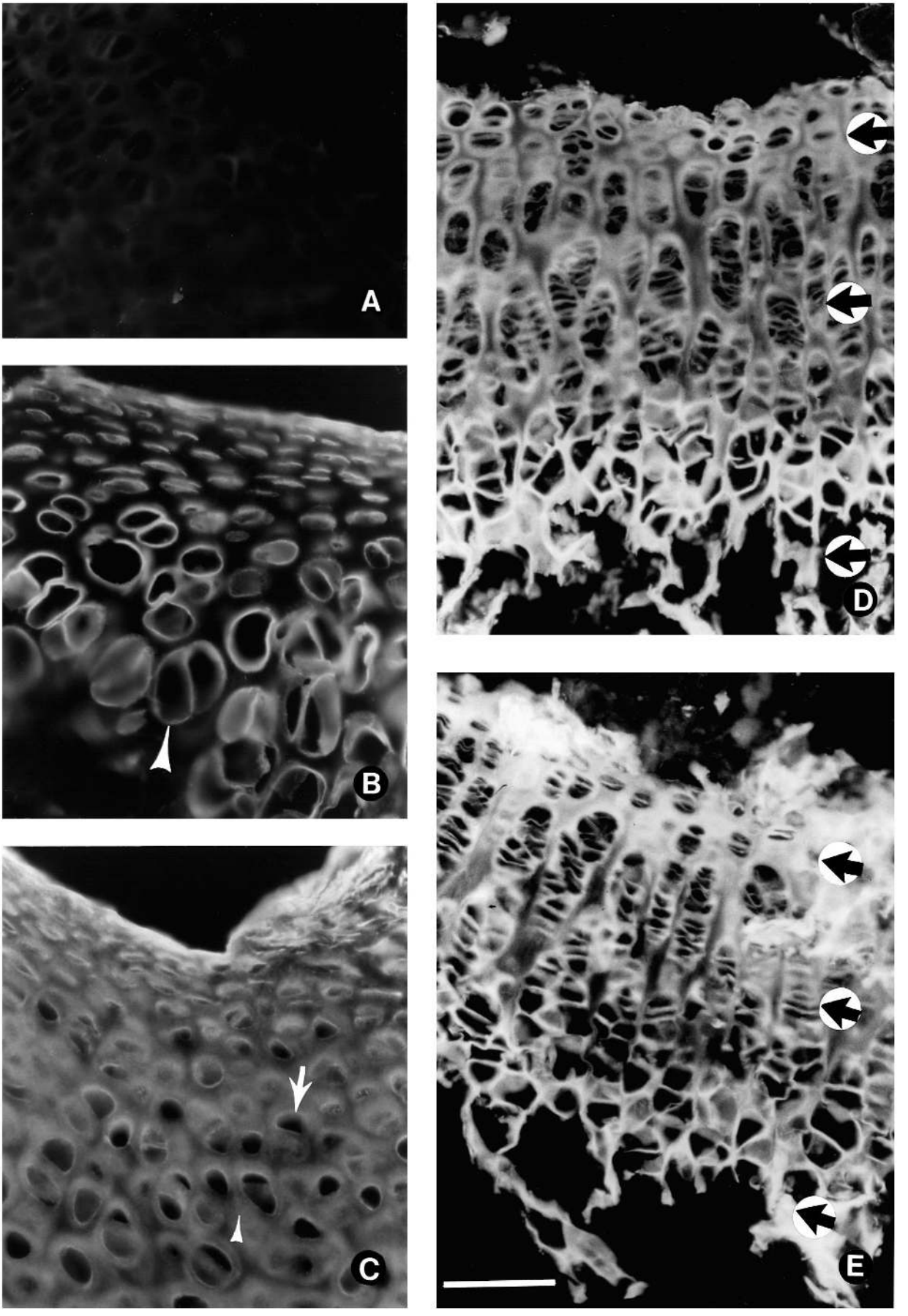


Fig. 5. Indirect immunofluorescence staining of day 15 embryonic chick sternum. Cryostat sections of the tissue were reacted with anti-perlecan (A-B), anti-type IV collagen (C-D), anti-aggrecan (E) antibodies or with preimmune serum (F). Intense cellular staining both for perlecan (A-B) and type IV collagen (C-D), a weak matrix staining for perlecan and intense staining throughout the matrix in aggrecan (E) are seen in these sections (A,C,E and F). Bars: (A,C,E,F) 100 μm; (B and D) 33 μm.


A
B
C
D
E

cartilage, weakly with territorial matrix and negligibly with the interterritorial matrix (Fig. 4A). Immunostaining for laminin (Fig. 4B) exhibited a similar pattern to that for perlecan. In contrast, type IV collagen was not detectable in the human nasal cartilage matrix (Fig. 4C). In the perichondrium, the basement membrane of the blood vessel did react with antibodies to type IV collagen (internal positive control), as well as with the anti-perlecan and anti-laminin antibodies. Immunostaining patterns for aggrecan, perlecan, laminin and type IV collagen in untreated and chondroitinase-treated sections were similar; however, chondroitinase treatment appeared to increase the intensities of staining with all of the antibodies. As expected, anti-aggrecan antibody reacted strongly with the entire cartilage matrix (not shown).

Immunostaining of embryonic day 15 chick sternal cartilage, both chondroitinase-treated and untreated, revealed a similar pattern (Fig. 5), but showed some differences from that of nasal cartilage. Antisera to perlecan stained the surface of the sternal chondrocytes intensely and also showed some staining of the extracellular matrix (Fig. 5A,B). In contrast to chondrocytes in human nasal cartilage, chondrocytes in the chick sternum reacted with antibodies to collagen IV (Fig. 5C,D). Antisera to aggrecan stained the interterritorial matrix, the territorial and the pericellular matrix so intensely that it was often difficult to determine the location of the chondrocytes (Fig. 5E).

Hyaline cartilage in the articular surface of the bone in the growth plate of the mouse exhibited slightly different patterns of distribution of perlecan and laminin than those of the nasal cartilage. While only the pericellular matrix and, to a lesser extent the territorial matrix, in the untreated sections reacted with the anti-perlecan (Fig. 6B) and anti-laminin antibodies (not shown), intense staining was evident also in the interterritorial matrix in the chondroitinase-treated sections (Fig. 6C). Staining for aggrecan, as expected, was seen in the territorial as well as interterritorial regions throughout the cartilage matrix on the articular surface (not shown) and in the growth plate (Fig. 6E). The staining extended all the way from the resting zone and the zone of hypertrophy to the region of calcification. Similarly, staining of chondroitinase-treated sections with anti-perlecan antibodies revealed that perlecan was present in the pericellular, territorial and interterritorial matrices not only in the articular cartilage but also throughout the growth plate from the resting zone to the zone of calcification (Fig. 6D). In contrast, immunostaining of type IV collagen was not detectable in the cartilage matrix in the articular surface or the growth plate (not shown).

Extracts of bovine articular cartilage were fractionated on a column of Sepharose CL-4B and individual fractions were assayed for aggrecan and perlecan content by dot blot using antisera to chick aggrecan and murine perlecan (Fig. 7). The perlecan-enriched fraction (tubes 18-22) was chromatographed on a DEAE-Sepharose column to separate proteoglycans from other glycoproteins. The immunoreactive material exclusively eluted in the proteoglycan fraction (not shown). The analyses of the proteoglycan fraction by SDS-PAGE and western blot with antisera to perlecan showed a faintly immunoactive band at ≈220 kDa (Fig. 8, lane 1). Digestion with heparatinase generated an immunoreactive band calculated to be ≈260 kDa (Fig. 8, lane 3). Digestion with chondroitinase alone did not generate additional bands that entered the gel (Fig. 8, lane 2). However, digestion with both heparatinase and chondroitinase generated an immunoreactive 260 kDa band, which was substantially darker than that seen with heparatinase digestion alone. These results indicated that, in the bovine articular cartilage, perlecan core protein may be proteolytically processed and it exists with heparan sulfate side-chains as well as with both heparan sulfate and chondroitin sulfate side-chains.

The pericellular location of laminin and perlecan suggests that they may serve as chondrocyte cell adhesion molecules. Consequently, we tested the ability of collagenase-digested IRC cells to attach to dishes (2 cm^2) coated with perlecan and laminin. Chondrocyte attachment on dishes coated with 10 μg of intact perlecan or perlecan core protein was 6-8% while on dishes coated with laminin at the same concentration it was 20% of the total cells plated (Fig. 9). Concentrations of laminin as high as 30 μg/dish did not increase cell attachment (data not shown). Although attachment to perlecan was only 6-8% of the total number of cells added, it is almost half as much as maximum attachment to laminin and significantly higher than

Fig. 6. Indirect immunofluorescence staining of bone from the tarsal joint of a 21 day mouse. Cryostat sections of the tissue were reacted either directly (A-B) or after treatment with chondroitinase (C-E), with polyclonal anti-perlecan (B-D) or anti-aggrecan (E) antibodies followed by FITC-conjugated secondary antibodies. In the articular surface of the bone sections stained without pretreatment with chondroitinase (B), only the pericellular region (arrowhead) stained intensely with anti-perlecan antibodies. In the chondroitinase-treated sections (C) not only the pericellular region but also the rest of the matrix including the territorial region (arrow) and interterritorial region (arrowhead) stained intensely with anti-perlecan antibodies. In the cartilage of the growth plate stained with anti-perlecan (D) and anti-aggrecan (E) antibodies, intense staining was evident throughout the entire matrix in the zones of resting cells (top arrow), of hyperproliferation (middle arrow) and of calcification (bottom arrow). Bar, 100 μm.

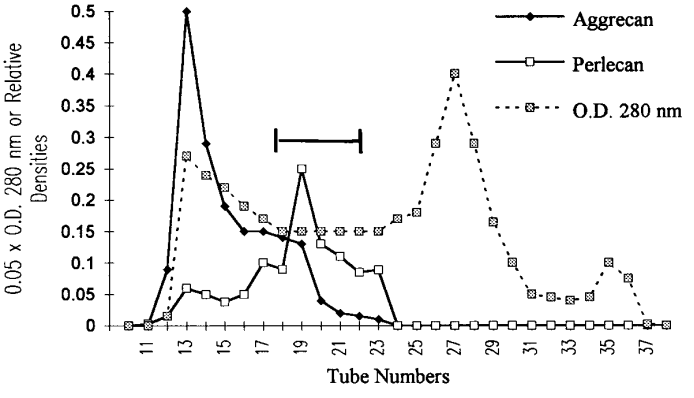


Fig. 7. Fractionation of bovine articular cartilage extract on Sepharose-CL 4B. A 4 M guanidine-HCl extract of articular cartilage was applied to Sepharose-CL 4B, and elution positions of aggrecan and perlecan were monitored by immuno-dot blot analyses of aliquots of each fraction using ECL (Amersham Life Sciences, Inc.) reagents. The relative densities of dots on the autoradiograms were evaluated by scanning densitometry using an image analyses system. The elution pattern of proteins was also monitored by absorbance at 280 nm. The fractions containing perlecan, indicated by the brackets, were pooled.

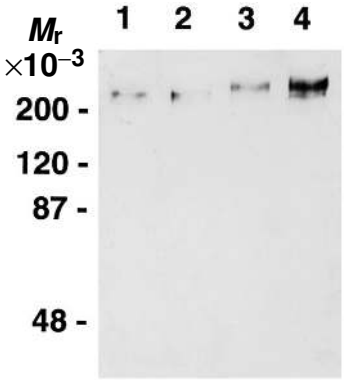


Fig. 8. Autoradiograms of the western blots of the perlecan-containing fraction from the bovine articular cartilage. Perlecan-containing fraction (tubes 18-22, Fig. 7) was further fractionated by DEAE-Sepharose chromatography and then subjected to western blot analyses, either directly (lane 1) or after digestion with chondroitinase (lane 2), heparatinase (lane 3) or both heparatinase and chondroitinase (lane 4). Treatment with ECL reagents (Amersham Life Sciences, Inc.) followed by autoradiography were employed for the detection of primary antibody binding. In the undigested sample (lane 1) a weak band is seen at the 220 kDa region. In the heparatinase-digested sample (lane 3) a darker band with approximate molecular mass of 260 kDa is detectable. In the chondroitinase-digested sample (lane 2) a weaker 220 kDa band is present. In the sample digested with both heparatinase and chondroitinase (lane 4), a doublet seen at 260 kDa. The band at 260 kDa is considerably darker than that seen in lane 3.

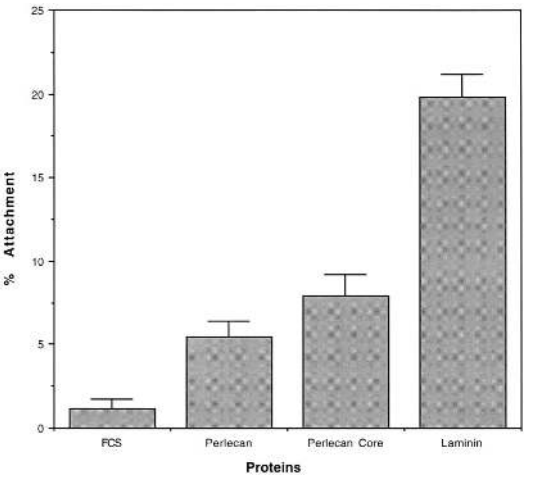


Fig. 9. Attachment of IRC cells to perlecan and laminin. IRC cells were biosynthetically labeled with [^{3}H]thymine and ≈50,000 labeled cells were plated per well of 24-well culture dishes coated with laminin, perlecan, or perlecan core protein (10 μg/well) and FBS (50 μl/well) as described in Materials and Methods. After 1½ hours incubation at 37°C, unattached cells were removed by rinsing the plates with PBS and radioactivity in the attached cells was counted after solubilizing the cells in the lysis buffer. The percentage cells attached was determined from the percentage of total counts in 50,000 cells. Each point is the average of four determinations and the brackets denote the standard error of the mean.

the basal level of 1% attachment obtained on dishes coated with 50 μl FCS.

DISCUSSION

The results of these studies show that chondrocytes make and secrete perlecan, both in vivo and in vitro. In studies in vitro, total RNA from IRC cells contained a 13 kb mRNA that hybridized to cDNA for all of the five domains of perlecan. Synthesis of the 400 kDa precursor protein for perlecan was detected in rat chondrosarcoma cells in culture, although it did not appear to receive extensive posttranslational modification. Perlecan extracted from bovine articular cartilage, however, contained heparan sulfate side-chains as well as both heparan sulfate and chondroitin sulfate side-chains. Furthermore, the core protein was only ≈260 kDa in size. Proteolytic fragments of the perlecan core protein were also detected in the glomerulus (Klein et al., 1988). Perlecan's large core protein has its 2-3 glycosaminoglycan side-chains attached to one end of the core and, because of this, a proteolytic clip in the middle of the core would produce a proteoglycan with approximately 200 kDa core protein.

Perlecan was originally isolated and characterized from the EHS tumor and was determined to be a heparan sulfate-containing proteoglycan. In native basement membranes, such as those produced by glomerular cells, perlecan contains heparan sulfate side-chains. On the other hand, a portion of perlecan synthesized by corneal fibroblasts is converted to a chondroitin sulfate proteoglycan (Hassell et al., 1992). Colon carcinoma cells produce perlecan primarily as a heparan sulfate proteoglycan, but a portion is produced with chondroitin sulfate side-chains as well, and a portion is produced without any side-chains (Iozzo and Hassell, 1989). Both heparan and chondroitin sulfate are attached to serine residues (S) followed by a glycine (G) residue, Not all SG sequences, however, have glycosaminoglycan (GAG) and there are likely other 'signals' on the core protein that influence this process (Hassell et al., 1993). It is also possible that the biosynthetic system available in different types of cells influences the GAG type made as well. Perlecan with different kinds of post-translational modifications may be involved in different types of cell-matrix interactions.

Immunostaining of hyaline cartilage tissues and chondrosarcoma demonstrated that perlecan is primarily deposited and retained in the pericellular or territorial matrix of chondrocytes in the nasal cartilage and chondrosarcoma. Perlecan was also deposited in the interterritorial matrix in the articular cartilage and in all the zones of growth plate during endochondral bone formation. Laminin also had similar distribution pattern to that of perlecan in these tissues but another basement membrane component, type IV collagen, was not detectable in these cartilage tissues. Collagen IV, however, was detected in the embryonic chick sternal chondrocytes. In contrast, aggrecan was detected in both the extracellular and the pericellular matrix of the chondrosarcoma and embryonic sterna. Perlecan in glomerular basement membrane contains heparan sulfate side-chains (Klein et al., 1988), which could act to perform the ionic filtration accomplished by the kidney. The large, multidomain core protein of perlecan, however, must perform other functions. The cell attachment properties of perlecan and laminin have been documented for a variety of fibroblastic, epithelial and endothelial cell types (Hayashi et

al., 1992; Graf et al., 1987). In the present study, we found that both perlecan and laminin act as cell attachment proteins for IRC cells at levels similar to that found for chondronectin and at levels considerably above that for fibronectin (Hewitt et al., 1980). Freshly isolated chondrocytes do not attach or attach very poorly to fibronectin-coated dishes (Hewitt et al., 1980, 1982) but after 10 days of culture in media containing 10% FBS up to 70% of the cells can attach to fibronectin-coated dishes (Loeser, 1993). Prolonged culture of chondrocytes in monolayer is well known to cause the cells to appear fibroblastic and cause changes in their phenotype (Benya and Brown, 1986). In the present study, we used primary cultures of IRC cells maintained in suspension, which retain their phenotype and, although they are less adhesive than chondrocytes grown in monolayer, they are more representative of the chondrocyte properties.

Cartilaginous tissues provide for the growth of long bones in development and act to absorb shock. These particular properties are primarily due to the compression of the aggrecan/link protein/hyaluronic acid complex. The compressive forces, however, are provided by another group of macromolecules that interact with each other and with the cell surface of chondrocytes. Denaturing solvents are required to extract perlecan from tissues, which indicates that perlecan's multidomain core interacts with other macromolecules. In cartilage, perlecan likely interacts with other adhesive macromolecules in the matrix to produce the compressed matrix, and with the chondrocytes to provide for cell adhesion to the matrix.

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