AN ANTISERA ALTERING RAT MID-FACIAL DEVELOPMENT REACTS WITH CARTILAGE PROTEOGLYCAN AND OTHER MATRIX PROTEINS

by

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An antisera altering rat mid-facial development reacts with cartilage proteoglycan and other matrix proteins.

Abstract

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In order to determine whether extracellular matrix proteins are involved in the transformation of nasal cartilage during growth and development, the present study was designed to assess the reactivity of a rabbit anti-rat nasal cartilage antisera (Hans, 1981) which causes midfacial abnormalities to occur in developing Sprague-Dawley rats.

Dot blot analysis, 3%-16% gradient SDS-PAGE electrophoresis, 2D-gel electrophoresis, and Western Blot analysis were used to identify reactive matrix antigens.

The protein patterns of nasal septum and condyle matrix proteins were grossly similar but some differences in quantity and quality between the two cartilage sites were identified.

The antisera was reactive with large proteoglycan and link protein. Small proteoglycans which contained dermatan sulfate by virtue of their reactivity with monoclonal antibody 2B6 after chondroitinase ABC digestion were also reactive, in addition to their reactivity with anti-nasal septum Ig G. Other specific monoclonal antibodies (8A4, 3B3, 5D4) were also used to characterize the reactive bands.

2D electrophoresis confirmed the results seen with 1D gels and in addition identified at least five proteins with the following pI,Mr: (6.9-7, 49-59), (6.85-6.92, 37), (6.4, 54), (6.2-6.3, 53), (6.1, 53), (5.9, 51), (5.9-6, 46). These have yet to be characterized.

This study provides the impetus for developing additional specific reagents (i.e. monoclonal antibodies) to define the potential extracellular matrix interactions required for normal nasal cartilage development.

DEDICATED
TO

My parents to whom I owe everything.

My wife Stephanie.

My brothers and sisters.

My country.

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INTRODUCTION

Therapeutic modification of facial growth has historically been one of the treatment goals of the clinical orthodontist. Because understanding the mechanisms that control facial growth is critical to the effective therapeutic modification of the process, craniofacial researchers have championed many different and sometimes competing theories of facial growth. The facial sutures, craniofacial cartilages, along with oronasal function have all been implicated at one time or another as primary centers controlling facial growth. In addition, the relative contributions of genotype along with environmental effects on phenotypic expression have also been debated. Advances in protein biochemistry as applied to general somatic growth demonstrated that hormones, chalones, and secondary messengers such as cyclic amp are also involved in the growth process.

Ultimately, control of growth in general and facial growth in particular must happen at the cellular level. Therefore, effective therapeutic modulation of the process would also be most effective at the cellular level. Arguably, the ability to modify the facial growth process at the molecular level to improve facial aesthetics and achieve facial balance would change the way clinical orthodontists practice. However, in order to achieve this goal more studies on the basic mechanisms that result in changes in cell activity (proliferation, quiescence, and senescence) are needed.

Cartilage is unique in the musculoskeletal system. Capable of both

appositional and interstitial growth with a pressure resistant extracellular matrix this tissue is ideally suited as an interface between growing bones. Because of the physical properties of cartilage early investigators hypothesized that the craniofacial cartilages (synchondrosal, nasal septal, and condylar) acted as centers for controlling growth of the cranial base, midface and mandible, respectively. Although the concept of "growth centers" has been largely disproven by extirpation and ablation experiments, the precise role of craniofacial cartilage in the growth process remains open to debate. Current facial growth theories generally recognize input from the genome, intrinsic and extrinsic growth factors, along with local and global environmental influences.

The first researcher to examine the possibility of protein differences among craniofacial growth cartilages was Brigham in 1976. Using indirect immunofluorescent techniques Brigham was able to detect antigenic differences (i.e. unique protein or proteins) between condylar, synchondrosal and nasal septal cartilage. The functional significance of these differences was not determined. However, Brigham and his coworkers speculated that these antigenic differences among cartilages may be used by the body to selectively control facial growth. Following up on Brigham's work, Palmer in 1978 injected growing rats with anti rat nasal septum cartilage antisera raised in rabbits. Palmer reported histologic evidence of cytopathic effects on the injected rats nasal septum chondrocytes. In 1981, Hans injected a similar anti-nasal septum antisera into growing rats from birth to 30 days. He observed a reduction in midfacial growth in the rat injected with the antisera.

These earlier studies were important because they allowed for the possibility of controlling facial growth by targeting individual growth cartilages within the face with specific antibodies. However, the specific proteins involved in growth modification seen with the anti-nasal septum antisera were not identified.

Recent advances in molecular biology have allowed the precise identification of proteins within certain tissues. In particular, new techniques allow researchers to separate the protein components of a particular tissue based on their molecular weight or charge. A separate set of techniques then allows antibodies directed against individual protein groups (Polyclonal) or even a single protein (monoclonal) to be used to identify specific proteins that may be found in a tissue such as cartilage. Because Hans had demonstrated that a polyclonal antisera could be used to change midfacial growth in the rat, the question remained as to what protein components were reacting with Hans' antisera. The proteins that reacted with Hans' antisera are most likely the proteins that are involved in controlling facial growth. It is to this end that this research was directed. Using rat nasal septum and condylar cartilage obtained from rats this study examined the protein structure of these two cartilages. After the protein components of the tissues were separated, the antisera used by Hans was tested against these proteins to determine which proteins reacted with the antisera. In addition to using the antinasal septum antisera, monoclonal antibodies directed against known structural proteins of cartilage will also be tested.

Using these biochemical techniques, the protein structure of two craniofacial cartilages, and the antigenicity of nasal septal cartilage will be studied.

LITERATURE REVIEW

Cartilage is a rigid and firm tissue and provides three basic functions. First, cartilage acts as a flexible support in various anatomic places (the nasal tip, ear lobe, thoracic cage, tracheal rings). Second, cartilage is a pressure tolerant tissue found in skeletal areas where compression occurs (articular cartilage). The third function of cartilage is as growth cartilage in conjunction with certain enlarging bones (synchondroses, condylar cartilage, epiphyseal plate, nasal septum)

The role of cartilage in the growth of bone has prompted researchers to manipulate cartilage in an attempt to control subsequent bone growth.

CARTILAGE HISTOLOGY AND BIOCHEMISTRY

Cartilage is an avascular connective tissue consisting of a large, highly organized, extracellular matrix which surrounds each chondrocyte. Hyaline cartilage is responsible for active growth in pressure areas by interstitial cell division of chondrocytes and by additions to its non-calcified intercellular matrix (Enlow, 1990). It is derived from condensation of mesenchymal cells.

The most important component of cartilage is water, approximately 60-80%. The remaining 20-40% of cartilage is composed of collagen, proteoglycans, cells, glycoproteins and lipids. The majority of the solid matrix is collagen, which makes up about 65% of the dry weight in articular

cartilage. Proteoglycans constitute another ≈25% of the dry weight, and the various other components of the solid phase make up the remaining fraction (Myers and Mow, 1983).

-Cells of cartilage:

Chondroblasts synthesize extracellular matrix. As chondroblasts divide and secrete matrix, cartilage expands and compresses the surrounding mesenchyme resulting in the development of a fibrous envelop called the perichondrium. The perichondrium has a tough fibrous outer layer and a cellular inner layer. The inner layer is made up of prechondroblasts which will later differentiate into chondroblasts. The deposition of perichondral chondroblasts is outward and results in surface apposition. These chondroblasts surround themselves with their own secretions and become chondrocytes. Chondrocytes can also divide and synthesize new matrix during growth, development or for repair.

Cartilage therefore, retains the potential to increase in size by **two** mechanisms:

1. Interstitial expansion.
2. Peripheral apposition.

Chondrocytes of mature hyaline cartilage are often seen in small clusters suggesting that some mitosis occur when the matrix is more rigid and unable to accommodate the separation of the daughter cells.

Although, chondrocytes like osteocytes reside in lacunae, they do not have processes which radiate out in canaliculi.

Two cell types are involved in cartilage resorption: multinuclear "chondroclasts" are responsible for resorption of calcified cartilage, and mononuclear cells are responsible for the resorption of uncalcified cartilage. While the multinuclear chondroclasts seem to have an origin similar to that of the osteoclasts, the origin of the mononuclear resorptive cell is still unresolved. While it is known that they are of perivascular origin, it is unclear whether they are derived from histiocytes, mesenchymal cells, macrophages, or monocytes. (Moss et al., 1983)

Collagens of cartilage:

Collagens consist of triple helical and globular protein domains mixed in various ways. Their role is predominantly structural reinforcement and their functional form is that of a fiber. Collagens have been designated by numbers (type I through XV) in chronological order of their molecular description. Another protein that is now considered a collagen, is presently called 1α 2α 3 α designating its three different chains. The nomenclature for designation of the collagens for a given collagen type with single chains is as α1, α2 and α 3, if three different chains are known, followed by the type number in parentheses. Thus, the chains of type I collagen are α1(I) and α2(I) and the molecule is [α1(I)]$_2$ α2(I). Collagen types II, IX, X and 1α 2α 3α are unique to cartilage.

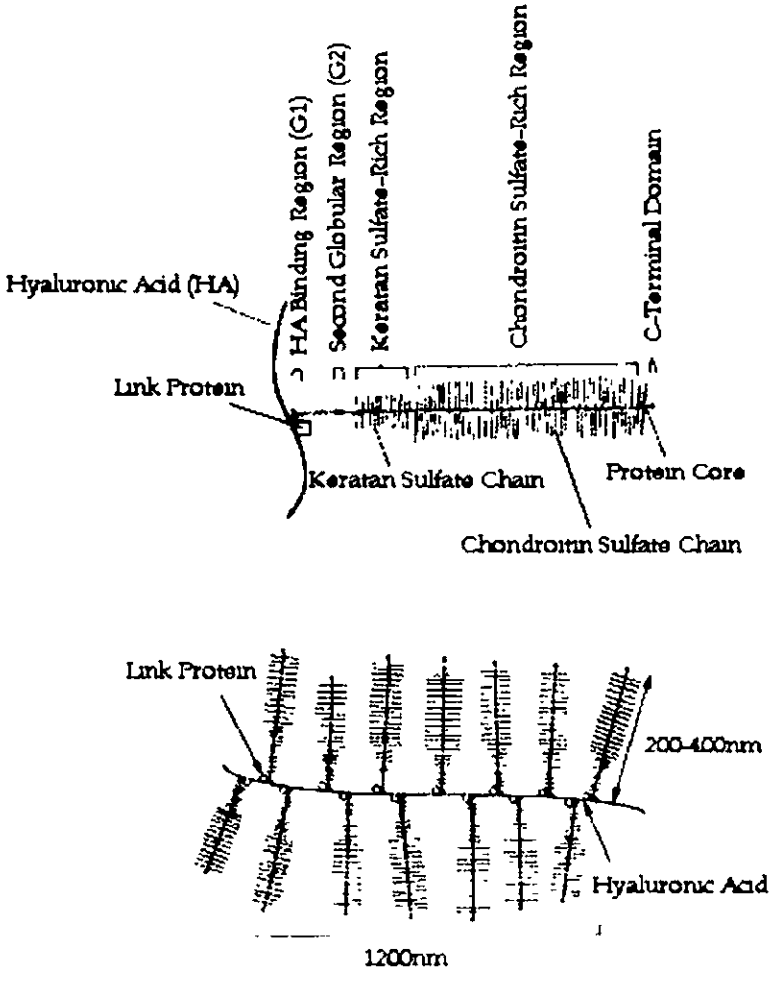
Type II collagen is the major collagen found in hyalin cartilage forming a three dimensional network of small diameter fibrils (Miller et al., 1976; Mayne, 1983). Aggregates of proteoglycans are entrapped within this meshwork, and the resiliency of cartilage is considered to arise from the

restraining effect of the collagen fibrils on these aggregates (Hascall and Hascall, 1981). Type II collagen is a homotrimer and has the formula [α1 (II)]$_3$, each chain has a molecular weight of 95 kilodaltons (Mr ~95 kD). In addition to cartilage, type II collagen has been identified in the vitrous humor of the eye and in the nucleous pulposes of the vertebral disk (Piez, 1987). These tissues are similar to cartilage in that they are avascular and have a high proteoglycan content.

Type IX collagen is found in cartilage but its function is unknown at the present time. Some investigators speculate that type IX could be involved in organizing and stabilizing the meshwork of type II collagen fibrils in hyaline cartilage. It consists of three triplets and has the formula [α1(IX) α2(IX) α 3(IX)]. In addition each chain has an Mr ~70 kD. It is both disulfide crosslinked and contains hydrolysine-derived crosslinks (Wu et al., 1984). Its aggregates are generally distributed throughout the cartilage matrix (Irwin et al., 1985).

Type X collagen is a short homotrimer, its formula is [α1(X)]$_3$. In addition to being unique to cartilage, it is synthesized only by hypertrophic cartilage. This has been confirmed by immunolocalization with monoclonal antibody (Schmid and Linsenmayer, 1985). Moreover, it was found that only the hypertrophic chondrocytes destined to calcify and to be replaced by bone initiate the synthesis of type X collagen (Gibson et al, 1984, 1985). This would suggest that Type X collagen is potentially involved in cartilage hypertrophy and calcification.

1α 2α 3α collagen has been identified as pepsin-resistant bands on gels having molecular weights of about 100 kD (Burgeson and Hollister,



Top: *Schematic representation of an aggregating proteoglycan monomer composed of keratan sulfate and chondroitin sulfate covalently bound to a protein core.*

Bottom: *representation of a proteoglycan aggregate composed of monomers non-covalently attached to hyaluronic acid, the interaction between the monomer and the hyaluronic acid core molecule being stabilized by link protein.*

(Mow et al., 1990)

1979). These bands apparently do not belong to other collagen. The matrix of hyaline cartilage does not appear to contain type V collagen and it is possible that the 1α, 2α, 3α collagens may function in cartilage in the same way that type V collagen functions in other tissues (Mayne et al, 1984). The 3α chain appears to be identical to α1(II) and differs only with respect to elevated levels of hydrolysine-linked carbohydrate moieties (Furoto and Miller, 1983). The 1α and 2α chains possess compositional features very similar to type V chains (Burgeson et al , 1982).

Cartilage proteoglycans:

Proteoglycans which are macromolecules with significant viscoelastic properties are also largely responsible for the swelling pressure of articular cartilage (Mow at al., 1984). This is due to the glycosaminoglycans whose structure gives them negative charges and allows them to bind large amounts of water. When cartilage is subjected to compressive force, water is forced out of the tissue. When the compressive force is removed, water is drawn back into the tissue by the glycosaminoglycans chains, and the tissue regains its shape. This capacity of the glycosaminoglycans to absorb water, combined with the structure and properties of the fibrous composite formed when the proteoglycans at high concentrations are enmeshed with and constrained by a dense network of collagen fibers, enhance the cartilage capacity to resist wear and damage caused by cyclical loading.

In cartilage, most of the 75-90% of proteoglycans exist in the form of proteoglycan aggregates formed by the noncovalent association of proteoglycans monomers, hyaluronate and link protein (Hascall, 1977). As

many as 100 proteoglycan monomers are bound to a single central hyaluronate filament via the hyaluronate-binding region of proteoglycan monomer core protein. The link protein appears to bind simultaneously to hyaluronate and to the hyaluronate-binding region of the proteoglycan monomer core protein. Link protein stabilizes the proteoglycan aggregate against dissociation. Aggregation of proteoglycans is a means of enhancing the concentration of non diffusible negative charges in the tissue (Kimura et al., 1986). In effect, the sulfate and carboxyl groups from the repeating disaccharide units of the glycosaminoglycans become charged in solution thus conferring a high concentration of negative charges to the proteoglycan molecules. These negative charges repel each other and bind water molecules, creating a high osmotic pressure, thus enhancing water absorption. *Kopp (1992) has emphasized the use of high molecular weight sodium hyaluronate for intra-articular injection in the treatment of some temporomandibular arthritis, especially cases with chronic* ***non****systemic arthritis of the TMJ. The high molecular weight sodium hyaluronate would favor the formation of larger proteoglycans aggregates, thus restoring soft tissue lubrication and reducing friction and pain.*

Proteoglycan **monomers** consist of chondroitin sulfate and keratan sulfate chains covalently bound to a protein core. The size of the core protein corresponds to M_r ~200 to 300 kD and exhibits considerable polydispersity (Heinegård et al., 1984). The protein core is composed of three main regions or domains. The portion of the protein core that extends peripherally in the proteoglycan aggregate is the C-terminal part which contains most of the chondroitin sulfate chains and is called the chondroitin-sulfate-rich region.

The chondroitin sulfate chains are attached through O-glycosidic covalent linkage to the hydroxyl groups of serine residues. The opposite end of the protein core, the N-terminal part, consists of a portion of the polypeptide chain that is essentially devoid of glycosaminoglycans chains. This region contains the binding site of the proteoglycan monomer for hyaluronate and a few N-linked oligosaccharides. This region is called the hyaluronate-binding region (Hascall and Kimura, 1982) which is about 60 kD in molecular weight and devoid of glycosaminoglycan chains. Between the chondroitin-sulfate rich region and the hyaluronate-binding region is located a third domain called the keratan-sulfate-rich region which is approximately 20-30 kD (Heinegard et al., 1977). This is the area where the keratan sulfate chains and a few O-linked oligosaccharides are attached to serine or threonine residues of the core protein through O-glycosidic bonds. Polydispersity in the newly synthesized proteoglycans is due to the variation in chondroitin-sulfate side chains (Fellini et al., 1984), while the core proteoglycan is monodisperse at this stage. With time and exposure to proteolytic activity in the tissue matrix, the core proteoglycan will decrease in size thus increasing in polydispersity.

Two populations of large aggregating proteoglycans are found in cartilage, showing discrete but clear differences with respect to size of protein core and proportions of side-chain substituents (Heinegard et al., 1985). These proteoglycans are best separated by agarose/polyacrylamide gel electrophoresis. They are characterized as having high contents of chondroitin sulfate chains with about 100 chains per proteoglycan molecule having a molecular weight of 18-20 kD. They also contain 40-50 keratan sulfate chains, 60-70 O-linked oligosaccharides, and 6 to 8 N-glycosidically

linked oligosaccharides. They are polydisperse, showing what appears as a continuous variation in molecular size expressed as broad bands on 1D gel electrophoresis. The molecular weights of the two types of aggregating proteoglycans are 3.5×10^6 and 1.3×10^6 respectively.

Large non-aggregating proteoglycans can also be found in cartilage. They appear to represent a distinct family with some structural similarities to the large aggregating proteoglycans (Heinegard et al., 1985).

Small proteoglycans exist in cartilage. Their molecular weight is less than 100 kD and they have a high content of aspartic acid, glutamic acid and leucine (Heinegard et al, 1981). The small proteoglycans from the nasal cartilage contain chondroitin sulfate chains with M_r ~36 kD, while those from the articular cartilage contain dermatan sulfate with a similarly high molecular weight. Also the core proteins are of similar size, M_r ~45 kD on SDS-PAGE after chondroitinase digestion, but differ when studied by peptide mapping.

Changes in proteoglycans with maturation and aging:

Some studies suggest that changes occur in the **chemical composition and size** of articular cartilage during maturation and aging. Specifically, these studies suggest there may be a progressive decrease in the length of chondroitin-sulfate-rich region of proteoglycan **monomer** with increasing age (Bayliss et al., 1983, 1984, Hascall and Kimura, 1982). Studies have compared proteoglycan monomers from fetal epiphyseal cartilage to proteoglycan monomers from mature articular and nasal cartilage. Based on sedimentation velocity and electron microscopic studies, fetal cartilage proteoglycan monomers were found to be more uniform in

length, and less polydisperse. Similar studies of proteoglycan monomers show that fetal epiphyseal cartilage and immature articular cartilages differ in **chemical** composition from monomers of mature articular cartilages. Fetal and immature articular cartilages contain more chondroitin sulfate, have longer chondroitin sulfate chains, and contain much less keratan sulfate. Controversy still exists about whether the increase in keratan sulfate content that occurs during postnatal maturation is due to keratan sulfate chain elongation or to an increase in the number of keratan sulfate chains (Sweet et al., 1979; Thonar et al., 1981). What is clear is that proteoglycan monomers from fetal and immature cartilage contain an undeveloped keratan-sulfate-rich region with a relatively low keratan sulfate content. It is also clear that the keratan sulfate content of this region increases during postnatal maturation. It has been suggested that the polydispersity, decrease in size, and changes in chemical composition of proteoglycan monomers that occur during postnatal maturation are most likely due to cleavage of the chondroitin-sulfate-rich region of the proteoglycan monomer core protein. This fragmentation of the chondroitin sulfate-rich region is probably due to proteolytic degradation of the core protein monomers which are thought to have resided in the extracellular matrix for a relatively long period of time (Rosenberg al. 1986).

Link Proteins:

The normal structure and special properties of mature articular cartilage and developing epiphyseal cartilage depend on the formation of large stable proteoglycan aggregates in extracellular matrix. It is these

aggregates that give cartilage its potent viscoelastic properties (Mow et al., 1984). Link proteins stabilize the binding of proteoglycan monomers to hyaluronate in these aggregates and increase aggregate size. The prevailing view is that each link protein contains an identical polypeptide chain into which are fused two binding sites. One binding site is for hyaluronate and one is for the hyaluronate-binding region of the proteoglycan monomers core protein . In the native aggregate, a molecule of one of the link proteins binds simultaneously to hyaluronate and to the hyaluronate-binding region to stabilize the aggregate (Rosenberg et al., 1986).

Mort, Poole and Roughley (1983) have shown that in some individuals, link protein is cleaved into fragments during aging. Using 4M guanidine containing protease inhibitors, link proteins were extracted from macroscopically normal human articular cartilages of different ages. The dissociative extracts were then subjected to equilibrium density gradient centrifugation under dissociative conditions (4M GdmCl). This process extracted both functional bound link protein and nonfunctional unbound link protein. Both types of link protein were concentrated with other matrix proteins in the low buoyant density fraction i.e. the top of the gradient. The proteins were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Proteins were then electrophoretically transferred from the gels to nitro-cellulose sheets. Link proteins were identified using a monospecific antiserum to link proteins, obtained from bovine nasal cartilage. Samples from fetal, neonatal, 2, 10, 22, 58 and 65-year-old human articular cartilage were then assayed. All samples contained three link proteins with molecular

weights of 48k, 44k, and 41k. However, mature and aging adult cartilages contain less 48K link protein and more 44K and 41K link proteins. Interestingly, under reducing conditions, additional link protein fragments ranging from 26K to 30K were present in the link protein preparations from mature and aging cartilages with the relative amounts of these fragments increasing with age. Under non-reducing conditions, the fragments could not be demonstrated by SDS-PAGE. These findings indicate that the link fragments are covalently bound to the 48K, 44K, or 41K link proteins by disulfide bonds. The authors suggested that fragmentation of link proteins results from limited proteolytic cleavage **in vivo**, which increases with age.

ANTIGENIC DETERMINANTS OF CARTILAGE

It has been known since the early days of modern plastic surgery that cartilage could be grafted between individuals to reconstitute cartilaginous defects, with little or no resorption. There was little difference in the short term results obtained using foreign (or allogeneic) cartilage and those using heterotopically transplanted autologous cartilage (Gibson et al., 1960). Although such grafts did enjoy some initial succes they invariably failed in the long term. Recent interest in the immunology of cartilage, and in particular articular cartilage, has been stimulated by the finding that the immune system is involved in diseases such as rhumatoid arthritis, other inflammatory arthritides, and relapsing polychondritis. All these diseases involve cartilage damage.

The immune response:

An immune response may ensue if a foreign material gains entry to the body. Three important requirements must be met. First, the host must be competent to mount an immune response. Second, the material must have antigenic sites on it in order to stimulate the immune system (i.e., have **antigenicity**). And third, those antigenic sites must be accesible or "visible" to the immune system (i.e., be **immunogenic**).

It is also important to distinguish between **immune** response and **inflammatory** response. In the latter case, phagocytes are the principal cells involved. Phagocytes destroy foreign materials inside or outside of the cell, by producing and secreting hydrolytic enzymes. Multiple exposures to the same foreign material stimulate new inflammatory responses. Each response is similar to the last, or to the response against an unrelated foreign body. In contrast, the immune response principally involves lymphocytes and other related cell types. The immune response also involves antibodies produced by plasma cells, "killer" lymphocytes, and a group of molecules collectively called the lymphokines. Although the inflammatory and immune responses can be separately described, reactions to immunogenic foreign materials usually have both inflammatory and immunologic components.

There are three components of cartilage that are possible sources of antigenic material: chondrocytes, collagen, and proteoglycans of the extracellular matrix.

Chondrocytic antigenicity

As integral parts of their surface membranes, the cells of most tissues contain a variety of antigenic sites. These antigens may be uniquely characteristic of that tissue (i.e., tissue-specific antigens), occur on all cells of the body but unique to that individual (i.e., individual-specific) or show common characteristics of all individuals of a given species (i.e. species-specific). Individual-specific antigens are most important when considering tissue transplantation. While tissue-specific antigens may be of importance in determining cellular differentiation having some role in the development of autoimmune disease or cancer.

Malseed and Heyner, 1976, used isolated chondrocytes from bone rudiments of fetal rats to immunize either rabbits (heterogenic) or an allogenic strain of rats. Cytotoxic antichondrocyte antibodies were detectable in the sera of immunized animals.

Antigens of the major histocompatibility system have been demonstrated on articular cartilage chondrocytes (Elves, 1974). Cells enzymatically isolated from sheep articular cartilage were used in cytotoxic assays with antisera against various antigens of the major sheep histocompatibility system. Lymphocytes from cartilage donor sheep were tested in parallel. There was a good correlation in the reaction with these antibodies in the antisera between chondrocytes and lymphocytes. The only difference observed was an increase in the time required by the antibody to kill chondrocytes compared to lymphocytes. This was almost certainly due to the presence of **adherent proteoglycan matrix** around the chondrocytes which impeded antibody access to the cell surface. Removing this remnant

matrix using enzymes (collagenase or trypsin) reduced the time required for serum to kill chondrocytes. These findings also demonstrated that certain antigens of interest were membrane bound and not matrix antigens.

In 1981, Glant et al. reacted chondrocytes from human, bovine, and canine articular cartilage with antisera against human and calf chondrocytes, human thymocytes, and bovine lymphocytes. These reactions showed the distribution of the tissue specific chondrocyte antigens over the entire cell surface. Bound antibody was visible as isolated particles on the side of microvilli. They also pointed out the existence of histocompatibility antigens on the cell membrane of human articular chondrocyte as well as the species-specific chondrocyte antigens on the surface of bovine articular chondrocytes.

In 1983, Burmester et al. used monoclonal antibodies and indirect immunofluorescence to identify surface antigens on chondrocytes. They pointed out the strong expression of Ia antigens on single cells lacking a pericellular matrix. In contrast, cells having an intact pericellular matrix did not have detectable Ia antigens. There was a good correlation between the occurence of Ia-antigen positive chondrocytes and the severity of the cartilage destruction as determined by gross appearance and histopathology. It was proposed that when cartilage cells are activated by a variety of stimuli, they may release enzymes capable of degrading their own local pericellular matrix. This altered state of the cell membrane revealed the presence of the Ia antigens.

In 1988, Mollenhauer et al. used sera from osteoarthritic patients to examine the immune reaction in inflammatory and degenerative cartilage

diseases. The immune reactivity of osteoarthritis sera was tested in a dot blot assay. Further, patient and control sera were tested in an Enzyme-Linked Immunosorbent Assay (ELISA), using chick cartilage cell membranes as substrate. Finally, the target antigens were characterized by western blot analysis of the membrane proteins. The authors reported that the predominant reaction was directed against cartilage cell surface epitopes

Potential antigens in collagen: (Elves, 1983)

The collagen molecule consists of three polypeptide chains (α chains) each of which is helically twisted on itself and is also twisted with the other two. Thus, the three α chains form a triple helix. There are at least five types of α chains, each of which has glycine in every third position in the amino acid sequence. This glycine is responsible for the coiled configuration of the molecule. The four major types of collagen found in cartilage tissues have unique α chain characteristics.

Collagen possesses at least three categories of antigenic sites. Sequential sites are found on the terminal non-helical regions. It is known that rabbits produce antibodies against bovine collagen. However, these same antibodies would not react with pepsin-treated collagen. Other experiments used cyanogen bromide (CNBr) to obtain peptides from both C- and N-terminal ends which have been shown to possess antigenic sites. Another feature of the non-helical terminal antigenic determinations is that they show little interspecies cross-reactivity i.e. they are species-specific.

A second type of antigenic site is found on the central portion of the α chain which does not depend on the two other α chains. Because helix formation masks the central portion antigens, experiments were conducted with denatured collagen and with α chains having the C- and N-terminal ends removed. Results of these studies demonstrated that the collagen molecules still reacted with the anticollagen antibodies. In contrast to sequential antigen sites, the central region α-chain antigens are not species-specific.

The third potential for antigens on the collagen molecule is the presence conformational sites on the intact triple helix. It was found that anti-

collagen antibodies raised in rats against calf collagen would not react with α chains separated by denaturation. If the chains were reassociated, the reactivity with the antibody was restored. If the wrong α chains were used to produce artificial helices, then they would not react with the antibody. Like central region antigenic sites, conformational (helical) antigens are species-specific and show little interspecies cross-reactivity.

These results demonstrated that collagen might have the potential to elicit an immune response in rheumatoid arthritis or other auto-immune diseases.

Antigenic sites located on proteoglycans:

The introduction of monoclonal antibody technology (Kohler & Milstein, 1975) has allowed investigators to examine the primary structural features of the proteoglycans. Monoclonal antibodies recognize single epitopes on complex molecules such as proteoglycans and allow specific studies even with the inherent heterogeneity and polydispersity.

Monoclonal antibodies can be used as analytic tools for both quantitative and qualitative analyses of proteoglycans. Using monoclonal antibodies, Dorfman et al., 1980, have shown the existence of at least two antigenic determinants in the core protein structure. Monoclonal antibodies directed against **carbohydrate etpitopes** on proteoglycans have been produced that specifically recognize each of the three isomers of chondroitin sulfate (0-, 4-, and 6-sulfated) and dermatan sulfate (Caterson et al., 1981; Couchman et al., 1984). These antibodies require that the glycosaminoglycan

chains be **pretreated** with **chondroitinase** in order to generate the **epitope** recognized by each monoclonal antibody. This finding showed that the antigens used for immunization were similarly pretreated, or the epitopes for the antibodies are normally sequestred in the proteoglycan aggregate interaction. All of the antibodies recognize an epitope containing an unsaturated glucuronic acid moiety at the non-reducing terminus of the carbohydrate chain. Differential digestion with either chondroitinase ABC or chondroitinase ACII prior to immunostaining can be used to distinguish between chondroitin-4-sulfate and dermatan-sulfate-containing proteoglycans (Couchman et al., 1984). **Radioimmunoassays** using monoclonal antibodies, in a sandwich (a second antibody coupled to an isotope is used to tag the monoclonal antibodies binding to the antigens), are then used to allow for qualitative and quantitative analysis. Monoclonal antibodies against epitopes present in the hyaluronic acid binding region, as well as the epitopes present on the link proteins, have been produced and used in peptide mapping of the proteoglycans (Caterson et al., 1986).

Affinity chromatography using monoclonal antibodies has been used for proteoglycan separation. The earliest successful use of monoclonal antibodies coupled with a beaded agarose column was in the purification of alpha interferon (Secher and Burke, 1980). Caterson et Al., 1986, used monoclonal affinity chromatography to demonstrate the occurence of two distinct proteoglycan subpopulations in young and old rat knee articular cartilage.

Monoclonal antibodies have also been used for topographical localisation of antigens. Burmester et al., 1983, used monoclonal antibodies

and **indirect immunofluorescence** for surface antigen identification on articular chondrocytes. They pointed out the presence and expression of Ia antigens on single cells lacking a pericellular matrix. The preceding studies demonstrate that proteoglycans may also be potentially unique antigenic sites within cartilage.

CONTROL FACTORS IN CHONDROGENESIS

Environmental regulation

Mature cartilage chondrocytes, embedded in extracellular cartilage matrix, are resting cells with no mitotic rates and a very low activity of matrix production and degradation. After enzymatic release from cartilage and transfer to cell culture, even chondrocytes from older individuals can be reactivated to divide and to produce cartilage matrix. Therefore, chondrocytes can be controlled by their microenvironment; the **cartilage matrix**. Studies of osteoarthrosis found that changes in the composition of the extracellular matrix, such as degradation and loss of proteoglycans, initiate chondrocyte proliferation and increase collagen and proteoglycan production in chondrocytes (Nimni et al., 1973). Furthermore, chondrocytes in culture maintain their **chondrogenic phenotype**, i.e. preserve their ability to produce cartilage-specific collagens. This occurs only if the culture conditions allow the cells to maintain a **spherical** cell shape and prevent spreading, outgrowth of cell processes and development of stress fibers (Benya et al., 1986). Certain culture conditions cause chondrocytes to spread

and to develop cell processes, focal contact and stress fibers. Chondrocytes seeded on tissue culture, plastic or glass (Dessau et Al., 1978), or those treated with hyaluronidase (Solursh et Al., 1975), can reject the pericellular matrix or "glycocalyx". Instead of matrix, these cultures deposit a fibronectin network that seems to develop an inhibitory effect on chondrogenesis. Such flattened and matrix-free chondrocytes are labile and may continue to produce cartilage-specific collagens and proteoglycans for some time. However, they will eventually lose these properties and begin to synthesize type I collagen and non-cartilage proteoglycans, eventually becoming fibroblastic or "dedifferentiated" chondrocytes.

Culture conditions that maintain chondrocyte phenotype include: culture on agar (Horwitz et al., 1973), in agarose (Benya et al., 1982), high density monolayer culture (Solursh et al., 1974), or high density suspension culture (Deshmuskh et al., 1976). The common feature of these culture conditions is the ability of the chondrocytes to encapsulate themselves in a pericellular matrix consisting of type II collagen and chondroitin sulfate proteoglycan (CSPG). This cultured cartilage matrix is apparently very similar to the native cartilage matrix. Von der Mark et al. (1986) showed that the binding of extracellular type II collagen to chondrocytes is mediated by a membrane bound glycoprotein of MW 34,000 daltons called **Anchorin CII**. The binding of radioactively labelled type II collagen to chondrocytes as well as the attachment of chondrocytes to type II collagen substrates is reduced, but not completely inhibited by antibodies to Anchorin CII. The same antibodies label Anchorin CII at the surface of chondrocytes in situ and in cell culture. The function of Anchorin CII is still unclear, but it might serve to

anchor the cell in the extracellular matrix without further consequences of the collagen-cell binding on cell functions. It could also be involved in endocytosis of collagen or secretion of procollagen. Anchorin CII may provide nucleation sites for the assembly of pericellular collagen fibrils or bundles as suggested by the punctate distribution of Anchorin CII on the chondrocyte surface.

Environmental effects on chondrogenesis

Caplan, A.I. ,1970, has found that in tissue culture, limb mesenchymal cells will readily **differentiate** into cartilage if the cells are plated at densities greater than confluency. Solursh et Al. ,1982, have shown that **single**, dissociated limb mesenchyme cells from chick embryos can be converted into chondrocytes. Chondrocytes are identified by the appearance of immunologically detectable type II collagen or the presence of an alcian-blue-stainable extracellular matrix. To stimulate conversion to chondrocytes, the limb mesenchyme cells are briefly maintained in suspension culture, cultured on or in agarose, methyl cellulose, or type I collagen gels, or briefly exposed to cytochalasin D (Zanetti et al., 1984). One noticeable feature of all these culture conditions is that they maintain the mesenchymal cells in a **rounded configuration** which is consistent with the normal role for cell shape in the initiation of chondrogenesis. Typically round shaped cells are found in the precartilage blastema *in situ*. Furthermore, localized differences in the **extracellular matrix** seem to play a role in establishing chondrogenic and non-chondrogenic regions (i.e. in **differentiation**). This is seen in the mesenchymal cells of the limb bud of chick embryos in situ. The normally non-chondrogenic regions of the early limb bud can form cartilage in explant

cultures and high density cell cultures (Ahrens et al., 1979). The addition of **limb ectoderm** appears to inhibit chondrogenesis and promote loose connective tissue formation (Solursh et al., 1981). In contrast, removal of limb ectoderm from the explant promotes more chondrogenesis. Its effects would be produced through the **extracellular matrix**, since collagen gels but not agarose gels can be preconditioned by ectoderm. Preconditioning is done by **alterating the shape** of mesenchymal cells. Cytochalasin D treatment alleviates the inhibitory effect of the ectoderm on chondrogenesis (Zanetti et al. 1986).

Modification of **cell shape** seems to influence the onset of chondrogenesis. This may be caused by altered gene expression through the cytoskeleton which in turn might influence the translation, and indirectly the transcription of specific messenger RNA (mRNA) populations. Zanetti et al., 1984 found that the actin skeleton is involved in controlling the shape of chick limb mesenchyme cell, Lenk et al., 1977 showed that the actin skeleton is intimately involved in mRNA translation. As mentioned, chondrocytes in culture maintain their **chondrogenic phenotype** only if certain culture conditions are met. Conditions must allow for the maintainance of a round cell shape, prevent spreading and outgrowth of cell processes, and prevent stress fibers from developing. Solursh et al. (1975) found that treatment of chondrocyte cultures with **hyaluronidase** removes the pericellular matrix, leading to cell flattening and reduced chondrogenic expression.

Modulation of the Chondrocyte phenotype *in vitro*

The collagen in cartilage is mostly type II. The remaining collagen is distributed between 1α, 2α, 3α-chain collagens, and type IX collagen. In growth cartilage, type X collagen appears during the hypertrophy stage. Most phenotypic modulation results in the cessation of type II collagen synthesis. Non-equivalent replacement of type II collagen occurs with synthesis of collagen type I or type I trimer, type III, type V and one or two uncharactarized smaller collagens (Benya et al., 1978). Thus, different experiments involving modulation of chondrocyte gene expression have been done *in vitro*. These in vitro studies include serial subculture (Benya et al., 1978), growth to senescence (Mayne et al., 1976), viral transformation (Gionti et al., 1983), growth in the presence of bromodeoxyuridine (BrdU) (Mayne et al., 1975), embryo extract (Mayne et al., 1976), retinoid acid (Benya et al., 1983), and phorbol myristate acetate (PMA) (Finer et al., 1984).

Modulation of chondrocyte phenotype has led to the following important findings. First, the process is **not** due to overgrowth by another cell type. This is clearly demonstrated by the fact that cultures derived from a single clonal chondrocyte exhibit the modulated phenotype at senescence (Mayne et al., 1976). Additionally, 80% of the modulated chondrocytes in masse culture and in fourth or fifth monolayer culture can be induced to reexpress the differentiated phenotype during subsequent agarose culture (Benya and Shaffer, 1982). Secondly, the cell morphology, which is polygonal or fibroblastic, does not necessarily correlate with the expressed collagen

phenotype. This has been shown by simultaneous immuno-localization of both collagen type I and II in the same field of partially modulated chick chondrocytes (Von der Mark et al., 1977).

Rexpression of the differentiated phenotype:

Subcultured-modulated chick chondrocytes were shown to reexpress the differentiated phenotype during a 2-week period of anchorage-independent agarose culture by Benya et al., 1982. One possible stimulus for reexpression could be the change in **cell shape** from spread to spherical. No changes were made in the culture environment or in the agarose medium to achieve a change in phenotypic expression. The change in shape from spread to **spherical** leads to a loss of stress fibers and to fewer spread cells, accompanied by a reorganization of the cellular cytoskeleton. It has been suggested that cytoskeletal reorganization (microfilament modification) might be involved in the transduction of shape information into interpretable signals for gene expression. This intracellular communication may occur by way of connections between the cytoskeleton and the nuclear matrix.

Phenotype modulation and reexpresion experiments have shown that chondrocyte phenotype *in vitro* can be highly variable. Phenotype expression could be modulated by powerful drugs and it may also be modulated by **subculture which** has been previously perceived as a relatively minor modification to the cellular environment. Another important possibility is that experimental designs *in vitro* that do not control for phenotype modulation

could be using cells that have **diverged** significantly from their *in vivo* state. In particular, the experimental requirements for essentially complete reexpression of a differentiated phenotype in chick chondrocytes include: microfilament modification, the presence of 10% fetal bovine serum, and the absence of a requirement for cell division (Benya et al., 1981).

Handley et al., 1986, showed that it is possible to maintain bovine articular cartilage, from both young and old animals, in culture for up to **3 weeks with no change** in the phenotype. The matrix surrounding the chondrocytes was also unchanged. When bovine cartilage is placed in a medium containing 20% fetal calf serum, the rate of S^{35}-labeled proteoglycan synthesis by bovine cartilage increases. The rate of loss of S^{35}-labeled proteoglycan from the tissue is low. The net result of these factors is the maintenance of the concentration of proteoglycans in the extracellular matrix. The primary role of serum in promoting tissue growth *in vitro* is to provide **hormones and hormone-like** factors. **Fetal calf serum** has been shown to contain activity corresponding to insulin-like growth factors or somatomedins (IGF/Sms). More precisely, IGF-I/SmC. IGF-I/SmC at 20ng/ml in the culture medium has the same effect as 20% fetal calf serum in the medium. Both substances dramatically increase the rate of synthesis and loss of proteoglycans.

Cartilage growth factors:

The proliferation and differentiation of animal cells are influenced by a variety of local substances, including a group of protein growth factors which are hormone-like in both structure and function. However, their synthesis, release, mechanism of action, and regulation are still unknown. The signal induced by most protein factors is mediated through specific cell surface receptors. These changes in cellular activities are associated with the cytoskeleton and therefore with morphological changes of the cell. Growth factors stimulate cell replication and metabolism .

1- Insulin-like growth factors (Somatomedins):

They include the insulin-like growth factors I and II (IGF-I, IGF-II) (Rinderknecht and Humbel, 1976), and the homologous rat protein, multiplication stimulating activity (MSA) (Moses et al., 1980). They are low molecular weight proteins (≈ 7.5kd) that circulate in plasma and they are also bound to high molecular weight carrier proteins (Zapf et al., 1975). In their bound form , the IGFs do not appear to have significant biological activity (Zapf et al., 1979). In vitro, they can be released from their carrier proteins by dissociating agents like guanidine or by acid treatment.

Cartilage derived factor (CDF) (Kato et al., 1980) was suggested to be most probably IGF-I (Kato et al., 1981b), whereas skeletal growth factor (SGF) (Kato et al. 1982) was believed to be IGF-II (Mohan et al., 1988). CDF was identified by its ability to stimulate ^{3}H-thymidine incorporation into DNA and ^{35}S-sulfate incorporation into proteoglycans by chondrocytes.

Interactions of CDF and MSA with other growth factors were evaluated. Both have synergistic activities with either EGF or FGF and DNA synthesis (Osborn et al., 1989). This is crucial since cells do not see only one growth factor at a time *in vivo*, and it is normal that several factors would act together to exert concerted biological activity.

Injecting IGF-I (100ng/day) into the growth plate of rats caused a significant increase in epiphyseal width (Russel and Spencer, 1985). Hypophysectomized rats showed a significant reduction in circulating IGF-I levels which were restored upon administration of growth hormone (GH) (D'Ercole et al., 1988). These results demonstrated the role of IGF-I in promoting cartilage growth, and also suggested that GH may act directly on chondrocytes to stimulate chondrocyte production of IGF-I.

2- Fibroblast growth factors:

Cartilage-derived growth factor (CDGF) which is distinct from the IGF-like cartilage derived factor (CDF) discussed above, is closely related to fibroblast growth factor (FGF). SDGF is able to bind tightly to heparin-Sepharose (Sullivan and Klagsbrun, 1985), and has a basic pI, and a molecular weight around 18,000 Daltons.

Sachs et al. (1982) found that FGF inhibited $^{35}SO_4$ incorporation in 48-hour cultures, but stimulated incorporation after 72-hour cultures. These findings suggest that treatment with FGF results in a short term decrease, but-long term increase in proteoglycan synthesis by isolated chondroblasts.

Other molecules related to FGFs are endothelial cell growth factors (ECGF) and macrophage-derived growth factor (MDGF). Both have been

shown to be mitogenic for osteoblasts and chondroblasts *in vitro* (Rifas et al., 1984). *In vivo* studies by Cuevas et al., 1988, demonstrated the potent effect of FGF on cartilage healing. FGF was administrated via minipumps to knees (≈ 200ng over 2 weeks) with intrachondrial lesions . After 20 days, the lesions were filled in with clusters of chondroblasts surrounded by extracellular matrix. The mechanism by which FGF exerts this healing response is not clear but FGF seems to stimulate both cell replication and matrix production *in vitro* as well as *in vivo*. FGF appears to be an attractive molecule to utilize in cartilage repair.

3- Transforming growth factor-β (TGF-β):

TGF-β is a 25 kd homodimer. It was originally studied as cartilage inducing factors A and B (CIF-A, CIF-B). CIF-A and CIF-B are now known to be identical to TGF-β1 and TGF-β2 respectively (Seyedin et al., 1986, 1987).

TGF-β1 and TGF-β2 have been reported to either stimulate or inhibit cell proliferation. Their action depends on the cell type, the experimental growth conditions and the presence of other biological factors (e.g. serum factors, Vivien et al., 1991). Skantze at al., 1985, demonstrated a decrease in both collagen and glycosaminoglycans production by chondroblast cultured with TGF-β in agarose culture. In contrast, Redini et al. (1988) found a stimulation of synthesis of both collagen and glycosaminoglycans. Synergism between TGF-β and EGF with mitogenic effects has also been observed in agarose culture (Skantze et al., 1985). O'Keefe et al., 1988, noted a biphasic mitogenic response of chick chondroblasts grown in monolayer when assays were done using Dulbecco's modified Eagle's medium (DMEM).

Recent studies by Morales et al., 1986, on the effects of TGF-β on organ cultures of bovine cartilage showed that TGF-β increased proteoglycan synthesis and inhibited catabolism of proteoglycans with no changes in the DNA or collagen content of the cultures. TGF-β1 has been shown to stimulate the synthesis of more anionic proteoglycan aggregates which presumably have a beneficial effect on tissue function as far as cartilage matrix resiliency is concerned (Morales et al., 1990).

Morales et al. (1991) showed that the predominant isoform of TGF-β is TGF-β1 (60-85%) and the total TGF-β1 + TGF-β2 was in excess of the amount required for maximal activation of proteoglycan synthesis. It was suggested that much abundance of the polypeptide might be sequestred in the matrix awaiting release upon demand.

4- Platelet-derived growth factors (PDGF):

PDGF is under the form of hetero and homodimer from 28-32 kd. It has been found to elicit a mitogenic response in vitro with increased $^{35}SO_4$ incorporation in chondroblasts (Prins et al., 1982).

5- Epidermal growth factors (EGF):

EGF is a 16kd monomer with potent mitogenic effects for chondroblasts (Hiraki et al., 1987) and which acts synergistically with FGF to stimulate DNA synthesis but at the same time reduce $^{35}SO_4$ incorporation in chondroblasts by both FGF and IGFs.

6- Interleukin-1 (IL-1):

IL-1 is a 17.5kd monomer. It has been associated with cartilage degeneration of diseased joints (Nowi et al., 1984). It has been shown to cause a decrease in $^{35}SO_4$ incorporation and type II collagen synthesis in chondroblasts, to provoke an increase in the release of proteoglycan from cartilage explants (Benton et al., 1988), and to stimulate prostaglandin production and secretion of proteases, including collagenase and elastase (Redini et al., 1988).

ROLE OF CARTILAGE IN FACIAL GROWTH

Growth cartilage plays a crucial role in facial growth. This cartilage functions in conjunction with certain enlarging bones in the form of synchondroses, condylar cartilage, epiphyseal plate, or nasal septum. Several theories have been developed to explain the close association between bone growth and cartilage. Early theorists believed craniofacial cartilages were primary growth centers. Later theories centered on cartilage as important growth sites in facial development.

Wolff's law of bone transformation, introduced in 1899, stated that bone adapts its structure to its functions. However, most attempts to apply Wolff's law have not distinguished between the physical forces acting on a bone and forces acting on the osteogenic connective tissue (periostum, growth cartilage). It is these later forces that actually form and remodel the

bone (Enlow, 1968). Enlow has also pointed out that areas of muscle attachment in the mandible are sometimes found on histologically resorptive bony surfaces. This finding would not be expected from Wolff's law.

Beginning in the 1930's, and flourishing in the 1940's and 1950's, a more complex model for growth control emerged. The new theory was based on the belief that growth of bone followed an **intrinsic genetic program**. The genetic program was stored by the bone producing cells of the periosteum, sutures, and related cartilages. Sutures, synchondroses, as well as the mandibular condyles were considered autonomous growth **centers**. These growth centers provided the tissue separating force that resulted in enlargment and displacement of bones. The downward and forward growth changes in the maxillary complex were thought to be due to the innate growth of the frontomaxillary, zygomaticomaxillary, and pyramido-sphenoid sutures (Weinman and Sicher, 1955).

The nasal septum theory (Scott, 1953), proposed that sutures are traction-adapted rather than pressure adapted. Scott suggested that the cartilagenous nasal septum provided the tissue separating force to displace the midface downward and forward. He believed that growth of maxillary complex as well as the adjacent organs was primarily driven by endochondral growth of the nasal septum.

Moss, 1962, 1968, developed the concept of a **Functional matrix** from the original concept of Functional Cranial Components of Van der Klaauw. In the Functional Matrix theory, the head and neck consist of independent yet integrated functions (i.e. digestion, respiration, speech, smell). Each function is carried out by a **functional cranial component**.

Each functional cranial component consists of a **functional matrix** and a **skeletal unit**. The mandible, for example, consists of several **independent** functional cranial components with associated functional matrix/skeletal units. The teeth and alveolar process are one such pair, the temporalis muscle and the coronoid process is another. The concept of a functional matrix is intended to describe what happens to achieve the combination of actions, eactions, and feedback interplay that occur. The functional matrix is primary while the associated skeletal unit is secondary. The skeletal unit is compensatory to the functional matrix which it supports and protects.

The composite concept, supported by Van Limborgh, 1972, surmised that growth control is achieved by a variety of factors. He proposed that intrinsic genetic factors, local epigenetic factors and environmental factors combine to control the facial growth process. In Van Limborgh's model, cartilage receives the most input from intrinsic genetic factors. In contrast, bone receives most of it modulation from local epigenetic factors such as hormones and local environmental factors such as oxygen. Cartilage and bone growth might generate epigenetic factors that will influence other surrounding cephalic structures.

USE OF CARTILAGE ANTIBODIES IN FACIAL GROWTH STUDIES

Early investigators have tried to verify the different theories of facial growth by surgical removal of craniofacial cartilages from experimental animals. Surgical trauma results in a destruction of the local environment and

introduces experimental error. It would be more elegant and convincing if the proposed cephalic cartilage could be rendered non-functional without surgical intervention. Anti-cartilage antibodies may be useful for that purpose.

Brigham (1976) developed antibodies in rabbits against bovine cephalic cartilage. Using indirect immunofluorescent labeling, he showed that antigenic differences exist between bovine condylar and nasal septum, or epiphyseal plate cartilages.

Working in the same laboratory, Palmer (1978) raised antibodies in rabbits against rat nasal septum components. Palmer injected growing rat from birth to 30 days with anti-RNS (rat nasal septum) antisera. He demonstrated that rat nasal septum antibodies are bound to the rat nasal septum cartilage *in vivo*. He also observed some histologic disturbances in the chondrocytes of the nasal septum which he termed cytopathic effects.

Hans (1981) also used a polyclonal antisera raised in rabbits to rat nasal septum cartilage extracts to inject growing rats from birth to 30 days. Hans showed that nasal septum antisera inhibits midfacial growth in rat. In addition, Hans developed an in vitro cell culture system to study anticartilage antibodies. Using nasal septum chondrocytes, Hans demonstrated that the nasal septum antisera was cytotoxic to chondrocytes in culture. Using indirect immunofluorescence, Hans showed that anti-nasal septum antisera localized to both the cell surface and the matrix of the cultured chondrocytes.

Perih (1989) prepared antibodies against **cultured** rat nasal septum chondrocytes and proved that in vivo and in vitro cell surface antigens and cartilage matrix antigens are for the most part identical in their immunofluorescent staining pattern.

Using SDS-PAGE electrophoresis and comparing the protein profiles of different cartilage extracts from whole cartilage and from cultured chondrocytes, Slodov, 1991, showed that unique proteins of different cartilages (nasal septum, condyle, epyphiseal plate) are most likely localised in the cartilage **matrix** rather than in the chondrocyte membranes.

Isolation and purification of proteins unique to cartilage and chondrocyte cephalic cartilage could be used for mouse immunization. These could then be used for hybridization, cloning and characterization of monoclonal antibodies directed against specific cartilage components. Monospecific anticartilage antibodies would be a powerful experimental tool. Thus antibodies could be used to begin to understand the complex mechanism involved in growth control.

STATEMENT OF THESIS

The first goal of this research was to determine the matrix antigens reactive with a cartilage antisera that alters the growth of the nasal septum in growing rats. This study will ultimately result in a better understanding of the factors affecting and controlling midfacial growth. Nasal septum matrix proteins will be reacted with this antisera that was shown previously to significantly alter the growth of the midface in 11-day rats (Hans, 1981). Dot blotting will be used as a preliminary exploration tool and to fine tune the analysis in order to characterize reactive species after 1D and 2D electrophoresis. Monospecific monoclonal antibodies (3B3, 2B6, 5D4, 8A4) will be used as probes to identify antigens common to these reagents and to the anti-nasal septum antisera.

The second goal of this study is to identify the matrix proteins components of 11-day nasal septum cartilage in relation with cartilage characteristics found in the literature.

The third goal of this study is to discriminate between nasal septum and condyle matrix antigens at the protein level. This would verify the results found by other investigators that existence of unique protein of nasal septum or condyle cartilage is likely possible.

Material and methods.

Sample dissection of cartilage:

Eleven litters of 11-day old Sprague-Dawley rats were sacrificed by Pentobarbital injection (2 ml). Access to the nasal septum was made by a longitudinal incision from nasion to the tip of the nose with a #15 blade. This incision was then emphasized by dissection with surgical scissors, starting from one of the nares exposed by slight amputation of the snout. Dissection pliers were used to separate the nasal septum from its base of insertion on the palatal bone. Excision of the condyle samples was much simpler. A lateral incision from the commissure of the mouth to the ear allowed access to the condylar head, which was the size of the tip of a pencil. The samples were then washed in cold Geys's Balanced Salt Solution (GBSS) to remove blood and surrounding tissue debris, then stored in Gey's solution at $4^{o}C$ until the end of the session. The conservation of the samples in cold isotonic media was crucial at this stage in order to minimize cartilage degradation by endogenous tissue proteinases.

At the end of each dissection, the samples were kept at $-75^{o}C$ for a few weeks before proceeding with tissue extraction.

Cartilage matrix extraction:

The following extraction strategy was employed for this investigation.

1-Freezing and milling:

The surface area of tissue material available for extraction can be increased by slicing, grinding or pulverising. Therefore, the dissected nasal septum and condylar cartilage samples were frozen with liquid nitrogen and milled into powder, using a Spex freezer/mill apparatus (Spex Industries Inc, NJ). During this manipulation, care was taken to place the impactor chip in the center of the sample tube and to avoid contact with the wet cartilage samples that would stick to the chip and would result in an incomplete milling.

2-Guanidine hydrochloride extraction:

Four M guanidine HCl in 0.1M sodium acetate buffer, pH 5.8 containing protease inhibitors (100 mM 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 10 mM Na_2 EDTA, 20µg/ml ovomucoid) was then added to the milling powder. The samples were thus left for extraction for 24 hrs on a magnetic stirrer at 4^{o}C. The extracts were then centrifuged for 10 minutes at 2,400 rpm (Sorvall J6-B) to eliminate any unextracted residue.

Salt solutions which promote solubilisation (as a consequence of dissociating intermolecular non-covalent interaction between proteoglycans, soluble collagens, glycoproteins and other molecules in connective tissues) have been termed dissociative extractants by Sadjera and Hascall (1969). They found that maximal extraction of proteoglycan (about 80% of tissue hexuronate) was obtained with high concentration of guanidine HCl; extraction at pH 5.8 resulted in optimal reaggregation after dilution of the guanidine HCl, and that a period of 20 hrs at 25^{o}C was optimal. The action of guanidinium salt is as a protein denaturant and its action on cartilage

proteoglycan complexes involves denaturation of link proteins (which are associated with proteoglycans) as well as denaturation of any secondary or tertiary structure which the proteoglycan subunit may contain. The viscosity of proteoglycan aggregates may be decreased by reduction with 2-mercaptoethanol, however this could inhibit the reaggregation of proteoglycans, link proteins and hyaluronate (Sadjera and Hascall, 1969).

3-Dialysis:

To remove guanidinium and to allow the proteoglycan aggreggates to reform, the extracts were then dialysed exhaustively against double distilled, deionized water for 24 hrs, at 4^{o}C, on magnetic stirrer, using porous membrane tubing with molecular weigh cut-off of 6,000 to 8,000 daltons. Water was changed every 6 hrs.

4- 15 K fractions:

A precipitate formed during dialysis. The dialysates were then centrifuged at 15,000 rpm (SS-34 rotor, Sorvall centrifuge), for 10 minutes to generate the 15-K supernate and the 15-K pellet fractions (precipitated material). These samples were assayed for hexuronic acid and protein.

5- Lyophilisation:

The 15-K supernatant (which contained most of the proteins) was frozen with dry ice in ether and lyophilized overnight. The 15-K pellet was kept at -4^{o}C for later comparison with other samples.

6- Cesium chloride density gradient separation:

The 15-K lyophilized supernatant was then solubilised in 12 ml of 4 M guanidinium HCl in 0.1M sodium acetate buffer, at pH 5.8, and centrifuged at 2,400 rpm for 10 min, yielding gradient pellets and gradient supernates. The gradient pellets were kept at -20°C . Cesium chloride was added to the gradient supernate (2.6 g of cesium chloride to every 4 ml of solution). The density was then adjusted to1.5± 0.0049 g/ml, using micropipets calibrated for 100µl and weighed on a precision balance (Mettler H20 analytical balance). The samples were then centrifuged at 35,000 rpm (gravity ≈ 105,000 g) for 45 hours at 4°C, yielding four density fractions D_1-D_4, bottom to top.

7- Dialysis:

To remove CsCl and guanidinium HCl, the extracts were then dialysed exhaustively against double distilled deionized water overnight, at 4°C, on magnetic stirrer, using porous membrane tubing with a molecular weigh cut-off of 6,000 to 8,000 daltons(Medical Spectrum Industries, LA, CA). Water was changed twice. This yielded the final four gradient fractions (D_1-D_4).

8-Uronic acid analysis and protein analysis:

Quantification assays were performed on different fractions: the 15-K supernates and pellets, the gradient supernates and pellets, and the final extracts.

Uronic acid analysis was performed to determined the concentration of proteoglycan. The carbazole method for uronic acid after Bitter and Muir (1962) was used.

Bradford micro-assay for protein was performed to quantify the amount of protein extracted in each fraction (Bradford, 1976).

Screening:

Further fractionation and characterisation of the extracts was done based on their molecular weight, charge and immunologic properties.

1- By molecular weight and charge:

1D(one dimensional) 3-16% gradient SDS-PAGE electrophoresis (Laemmli procedure) stained with Coomassie blue and silver nitrate allowed for separation based on molecular weight and comparison of nasal septum and condyle protein patterns.

2D 3-16% gradient SDS-PAGE gels (O'Farrell procedure) was then performed to discriminate between proteins migrating with poor resolution on 1-D gels. Additional resolution was obtained by the addition of reducing agent (1mM of dithiothreitol) to break the disulfide bonds.

2- By immuno-reaction:

Dot blot analysis was used to probe the different fractions, especially D_3 and D_4, with rabbit antirat polyclonal antisera (Hans, 1981) raised against

rat nasal septum and condyle , and with monoclonal antibody 8A4 (specific to link protein of proteoglycan).

Western blot analysis with specific rabbit anti-rat polyclonal antisera (anti-NS IgG and anti-CON IgG) and monoclonal antibodies (8A4, 3B3, 2B6, 5D4) was used for further characterisation of matrix proteins on 1D and 2D gels. 8A4 which is specific to link protein was used as a positive reaction control.

Anti-NS IgG and anti-CON IgG were extracted from antirat polyclonal antisera (Hans, 1981) by selective elution from DEAE-cellulose column (Whatman DE-52) following the technique described by Mac Donough and Inman (1970).

Comments:

1-Extraction conditions:

The surface area of tissue available for extraction can be increased by slicing, grinding or pulverising. Sadjera and Hascall (1969) used a Stanley surform. Brandt and Muir (1971) pulverised cartilage by hammering in a steel die cooled in liquid nitrogen. Prolonged high speed homogenisation of bovine cartilage in water permits extraction of 80% of the proteoglycan (Sandson et al., 1966), however the material extracted this way is degraded. Furthermore, proteases active at neutral pH have also been found in cartilage proteoglycans prepared by high speed homogenisation. Sonication is another technique that has been used, but it may, in addition to creating shearing forces, give rise to free radicals which could degrade protein or carbohydrate moities of proteoglycans. As shown by Sadjera and Hascall

(1969), very marked reduction of sedimentation value (**s**) occurs on sonification of proteoglycans, thus this technique should be avoided if intact proteoglycans are to be isolated.

2-Extractants:

Exposure of embryonic cartilage to water has been found to result in the release of lysosomal proteases (Lucy et al., 1961). Thus, it is preferable to avoid hypotonic conditions and protease inhibitors should be included during extraction.

Various substances have been used to extract the matrix proteins: urea, detergents (Triton X-100, SDS), salt (potassium salt, lithium salt), organic solvents (chloroform-methanol, aqueous butanol..), and proteolytic enzymes. The predominant problem encountered in using these substances was low extraction yield or denaturation of the proteins.

A breakthrough came when Sadjera and Hascall (1969) introduced extraction with chaotropic solvents like guanidine HCl combined with purification by sedimentation equilibrium centrifugation in CsCl gradients. This technique provided a high degree of separation of proteoglycans and matrix proteins. The authors observed that there was specific aggregation of cartilage proteoglycans to much larger structures and that guanidine HCl induced the dissociation of such aggregates present in the tissue. This dissociation facilitated the extraction of the proteoglycans from the cartilage. When the guanidine HCl was removed by dialysis, the aggregates reformed. A protein factor with a higher buoyant density than the proteoglycans in CsCl was required for the reaggregation. Keiser et al. (1972), later characterized

a class of proteins involved in aggregation that actually contained three similar components with apparent molecular weight of about 45,000. They are now known as **link proteins**. Hardingham and Muir (1972) showed that a third component, **hyaluronic acid**, could mediate aggregation by binding several proteoglycans. It was found that link proteins appeared to be an important factor in stabilizing the interaction between proteoglycan and hyaluronic acid (Hardingham,1979).

Sadjera and Hascall (1969) determined that 4M guanidinium HCl is the preferred concentration for extraction, since at this concentration most intermolecular non-covalents bonds appear to be broken. At lower concentrations, the denaturing effect of the guanidine hydrochloride is less pronouced and extraction is less efficient At higher concentration, the extraction yield may become lower, possibly an effect in the alteration of the collagen network as a result of denaturation. The ratio of extraction solvent to tissue wet weight should be kept at 15 to 20, to give optimal yield. It is important to use as small pieces of tissue as possible , to minimize extraction time. The optimal pH was found around 5.8, where the activity of lysosomal acid proteases as well as neutral proteases is low . A number of protease inhibitors have also been included in extraction solvents. Examples include the serine protease inhibitors diisopropyl fluorophospate (5 mM) and phenylmethylsulfonyl fluoride (1mM) which should be dissolved in small volumes of organic solvent, for instance n-propanol before addition. Other suitable inhibitors (Oegema et al., 1975) include 10mM benzamidine hydrochloride (inhibits arylsulfatases), 50 mM EDTA (inhibits metalloproteases), 100 mM 6-aminohexanoic acid (inhibits serine proteinases

such as plasmin and its activators) and 10 mM N-ethylmaleimide (NEM) (inhibits sulfhydryl-dependent proteases) which also prevents disulfide exchange. The addition of reducing agents such as 2-mercaptoethanol or dithiothreitol might help to solubilize microfillar component and therefore yield additional material, however reaggregation of proteoglycans, link proteins and hyaluronate could be inhibited (Sadjera and Hascall, 1969).

3- Cesium chloride density gradient centrifugation:

This technique separates molecules of different buoyant density. Before centrifugation, solid CsCl is added directly to the 4M guanidine hydrochloride extract or homogenate. Guanidine hydrochloride promotes solubilisation by dissociation of intermolecular interaction between highly charged proteoglycans and other molecules in connective tissue (Sadjera and Hascall, 1968). The starting density is adjusted between 1.35 to 1.50 g/ml, depending on the type of proteoglycan and preparation that is to be fractionated.

The amount of CsCl to be added to 4M guanidine extract to obtain certain density is calculated from the equation:

$$CsCl\ (g/ml) = 1.3468\ \rho - 1.4750$$

ρ is the desired density in grams per milliliter, which may vary depending on the purity of CsCl used. Usually, addition of 0.7 g CsCl (analytical grade) to 1 g of extract gives a density of 1.58 g/ml, while addition of 0.35 g of CsCl /g of extract gives a density of about 1.35 g/ml.

Centrifugation should be performed at 100,000 g or higher for an extended period (24-96 hrs). Steeper gradient and faster equilibration times are obtained with higher speeds.

In this present study, we used the Beckman SW 50.1 rotor (83.5mm), on L8-55 Beckman ultracentrifuge, at 35,000 rpm (115,000 g) for 45 hrs.

As described by Heinegard et al (1987), after centrifugation small proteoglycans (PG-Sm) will be recovered at densities between 1.30 and 1.40 g/ml in 4M guanidine Hydrochloride. Heparan sulfate proteoglycans from basement membrane show bands at 1.3-1.4 g/ml (low density) and at densities above 1.55 g/ml (high density). Large proteoglycans have high buoyant densities and those from cartilage will sediment at the bottom of the tube using various concentrations of CsCl. Proteins will be recovered at the top of gradients of most density ranges. Glycoproteins having a large number of negatively charge oligosaccharide chains will have a somewhat higher buoyancy density than most proteins and are recovered between the bottom and top fractions. Proteoglycans containing heparan sulfate chains or proteoglycans of the small type containing few galactosaminoglycan chains have low buoyant densities, the heparan sulfate ones usually having the lowest buoyancy. Hyaluronate will band in in 4M guanidine HCl at densities of 1.40-1.50 g/ml.

After centrifugation, the gradient can be emptied using a Beckman tube slicer and by upward displacement, with the tube into 4 fractions. Densities of different fractions (D_1-D_4, bottom to top) can be measured by pycnometry, using constriction pipets which are calibrated for 100 µl weighed on a precision balance.

Proteoglycans prepared in gradients containing 4M guanidine HCl are usually referred to as D (dissociative), D_1 is the bottom fraction. Those prepared in gradients with non-denaturing conditions where proteoglycans aggregates can be recovered (e.g. dialysis to 0.4M guanidine HCl) are referred to as A (associative). Thus $A_1.D_1$ is a proteoglycan purified in two sequential gradients, the first associative and the second dissociative.

4- Uronic acid analysis:

The uronic acids in proteoglycans are D-glucuronic acid and L-iduronic acid. One or both may be present in a single polysaccharide chain. Keratan sulfate and animal glycoprotein (with a few exceptions) do not contain uronic acid. Therefore assays for uronic acid can be used for the **detection of most proteoglycans** as well as for the analysis of their composition. However, with extracts from major tissues, a major problem is the presence of large proportions of nucleic acids which can band from very high to intermediate densities in CsCl gradients. Unfortunately they react both in the carbazole and in the orcinol procedures to give color (Beeley, 1985).

We have used the Carbazole method for uronic acid after Bitter and Muir (1962) with some variations: sodium borate (for color stabilisation), 0.025M, 1250µl is dissolved in 1250µl of concentrated sulfuric acid (analytical grade) for every tube. To avoid losses due to excess heat formed when water and sulfuric acid mix, the latter should be cooled, preferably on dry ice before the assay, or the tubes should be on ice during the mixing. Then for the standard tubes, 200µl of standard solutions (20-200µmoles uronic acid) is added to each. For each sample tube add 50µl of sample and 150µl distilled

demineralised H_20, thus the samples are 1/4 diluted. For the blank just add 200µl distilled demineralised H_20. The tubes were heated in a boiling water bath for 10 min then cooled to room temperature. Carbazole reagent, with 100µl of 0.125% Carbazole in absolute ethanol was added. The resulting solution is then heated for 15 min in a boiling water bath and allowed to cool to room temperature. The absorbance is read at 530 nm.

The absorbance of the blank read against sulfuric acid should be below 0.025. Dermatan sulphate or iduronic acid derivatives give approximatively 80% of the colour obtained from an equivalent amount of glucuronic acid. Neutral sugars give some colour in the assay when present in large amounts. Oxidizing agents (e.g. nitrites) give a green colour which interferes in the assay.

5- Bradford microassay for protein (Bradford, 1976):

Total volume of each tube is 1ml.

1) Add to each tube 200µl of dye concentrate (Bio-Rad Protein assay, cat.500-0006).

2) For Blank tube, add 800µl of ddH_20. Vortex.

3) For Sample tube, add 50µl of sample and 750µl of ddH_20. Vortex.

4) For Standards tubes, Bovine serum albumine (BSA) was prepared at 100µg/ml at kept at 4°C. The standards tubes will be loaded following this chart.

STDS (BSA in µg)	µl (of 100µg/ml BSA)	ddH_20 in µl
1	10	790
2	20	780

4	40	760
6	60	740
8	80	720
10	100	700
12	120	680
14	140	660

5) Read optical density (O.D.) after 5 min, on spectrophotometer at 595 nm, visible light.

6) Compute standard regression function.

7) From standard regression function, compute sample protein for 50µl and deduct sample concentration.

6- IgG purification from rabbit anti-rat polyclonal antisera:

We followed the procedure of Mac Donough and Inman (1969). Their technique utilizes ion-exchange material, DEAE-cellulose, for separation of the serum immunoglobulins.

1) Proteins in rabbit antisera were precipitated with saturated ammonium sulfate (antisera/ammonium sulfate, volume per volume); this causes most of the immunoglobulins to become insoluble and leaves albumin in solution.

2) The precipitate was centrifuged at 8000 g for 10 min, using a Sorvall RC-5B, rotor SS-34.

3) The precipitate was resuspended in a volume of about 20% that of the original volume.

4) In order to remove the ammonium sulfate, the material was dialyzed in porous membrane tubing with 6000-8000 molecular weight cut-off, overnight against 0.0175M sodium phosphate buffer, pH 6.9.

5) The dialysate was fractionated by elution from DEA-cellulose column (DE-52). Fractions were eluted with 0.0175M sodium phosphate buffer, pH 6.9 and spectrophotometrically monitored at 280 nm until the elution passed the first peak, peak A, described by Mc Donough and Inman as containing mostly Ig G's.

6) The Ig G's fractions were then assayed for protein concentration through the Bradford micro-assay.

7- Electrophoresis:

(Hoefer Scientific Instruments, Techniques and exercises,1991)

Electrophoresis is used to study the properties of single charged species and also to separate charged molecules in solution, chiefly proteins and nucleic acids, based on their migration response to an electrical field.

In electrophoresis, one electrical parameter (current, voltage or power) is always held constant. Most of the time, the resistance increases with the run with the following consequences, depending on the constant mode selected. In constant current, the velocity is maintained (velocity is directly proportional to current) but heat is generated ($P = I^2R$, P is power in watts, I is intensity in amperes, R is resistance in ohms). In constant voltage, the velocity slows, but no additional heat is generated during the course of the run ($I = E/R$ and $P = EI$, E is voltage in volts). In constant power, the velocity slows but heating is kept constant. Sample diffusion and loss of activity

caused by heat or time can thus be minimized by modifying these parameters.

The net charge of proteins is determined by the pH of the medium. Proteins are amphoteric compounds, i.e. containing both acidic and basic residues. Their net charge is determined by the pH of the medium they are in. Most of the charge of the proteins comes from the pH dependent ionization of carboxyl and amino groups:

$COOH^- \rightleftharpoons COO^- + H^+$ $\qquad$ $NH_2^- + H^+ \rightleftharpoons NH_3^+$

Therefore, each protein has its own characteristic charge properties depending on the number and kinds of amino acids carrying amino or carboxyl groups. It must be emphasized that nucleic acids, unlike proteins, are not amphoteric. They remain negative at any pH used for electrophoresis.

There is a pH at which there is no net charge on a protein: this pH is called the isoelectric point or pI. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates toward the anode in an electric field. Below its isoelectric point, a protein is positive and migrates toward the cathode. Thus, the pH of a solution in an electrophoresis system must be kept constant to maintain the charge, and, hence, the mobilities of the proteins. To accomplish this requires the used of buffered solution in electrophoresis.

Temperature regulation is another factor to consider. Polymerisation of the gels is exothermic and if not regulated, it will set up convections that cause irregularities in the pore size of the gel. Gels should be surrounded by a liquid coolant held at a constant temperature. During electrophoresis,

constant temperature is essential to avoid denaturing heat labile proteins and variation in velocity between samples in the center and the edges of the gel, giving a "smile" effect and making the comparison of bands difficult. For precise regulation, the electrophoresis tank must include a system for cooling and circulating buffer, with the lower buffer chamber large enough to allow the gel tubes or sandwiches to be immersed in temperature regulated buffer.

The most commonly used support matrices are agarose and polyacrylamide both of which make porous gels which act like a sieve. This is accomplish by retarding, or in some cases, by completely obstructing the movement of macromolecules while allowing smaller molecules to migrate freely. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids and large proteins and protein complexes. Polyacrylamide, which makes a small pore gel, is used to separate most proteins and small oligonucleotides.

The pore size in agarose gels may be predetermined by adjusting the concentration of agarose in gel: the higher the concentration, the smaller the pore size. Working concentrations are frequently in the range of 0.4 to 2% w/v. In polyacrylamide gels, the long chains are covalently linked by a crosslinker. The most common one is N'-methylene-bis-acrylamide, or *bis* for short. The pore size in a polyacrylamide gel may be predetermined in either two ways. One way is to adjust the total percentage of acrylamide, that is the sum of the weights of the acrylamide monomer and the cross-linker. This is expressed as %T (e.g. a 20 %T gel would contain 20 % w/v of acrylamide plus bis). As the %T increases, the pore size decreases. The other way to adjust pore size is to adjust the amount of crosslinker, expressed as the

percent of monomer and crosslinker or %C. Thus a 20%T 5%C gel would have 20% w/v of acrylamide plus bis, and the bis would account for 5% of the total weight of the acrylamide. It has been found that at any single %T, 5% crosslinking creates the smallest pores in a gel. Above and below 5%, the pore size increases.

When there is a wide range in the molecular weights of the material under study, the researcher may prepare a pore gradient gel, where the pore size is larger at the top of the gel than at the bottom. The gel becomes more restrictive as the run progresses. This enable proteins of widely different sizes, which would otherwise require runs at two or more different values of T, to be separated on a single gel, and particularly superior separation of relatively small components.

Polyacrylamide gel is chemically complex, and so also is the production and the use of the gel, with some technical requirements having to be observed. Because oxygen inhibits polymerization, the monomer mixture must be deaerated. This may be done by purging the mixture with an inert gas or by evacuating the mixture with a vacuum. When the gel is poured, either in a tube or in a slab, the top of the gel forms a meniscus. If ignored, this will cause a distortion of the banding pattern. To eliminate the meniscus, a thin layer of water or water saturated n-butanol is carefully floated on the surface of the gel mixture before it polymerizes. After polymerisation, the water or butanol layer is poured off, leaving a flat upper surface on the gel. This layer of water or n-butanol excludes oxygen that would inhibit polymerisation on the cell surface.

Polymerisation of a polyacrylamide gel is accomplished either by a chemical or a photochemical method. The photochemical method is used when a lower ionic strength is to be maintained in the gel. It is also used when the protein studied is sensitive to ammonium persulfate or the by-products of chemical polymerisation. Besides these special considerations, chemical polymerisation with ammonium persulfate and the quaternary amine, N, N, N', N'-tetramethylethylenediamine or TEMED, are used as the initiator and the catalyst respectively. To prevent excessive heating, the concentration of initiator-catalyst should be adjusted to complete polymerisation in 20 to 60 minutes.

In 1D SDS-PAGE electrophoresis, separation and migration is determined not by intrinsic electrical charge of polypeptides, but by molecular weight . The technique was introduced by Shapiro et al. in 1967. When proteins are heated to 100^{o}C for 2-5 min in the presence of SDS (sodium dodecylsulfate) and reducing agents, they unfold and bind ~ 1.4 grams SDS per gram of protein, approximately one SDS molecule for each two amino acids. The exact mechanism of binding is still poorly understood. SDS is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone, giving a net strong negative charge to the proteins, in proportion to their length, and dominating their native charge. Thus treated, the polypeptides become rods of negative charges with equal charge densities or charge per unit length. A linear relationship exist between the $\log_{10}$ of the molecular weight of a polypeptide and its R_f, which is the distance from the top of the gel to the polypeptide divided by the distance from the top of the gel to the dye front (Andersson et al. (1972),

Lorentz(1976)). A standard curve can thus be generated and would allow to determine the molecular weight of the studied proteins:

$$\log_{10}MW = A\,R_f + B$$

A and B are the slope and the intercept respectively of the linear regression line.

2D gel electrophoresis is used to separate complex mixtures of proteins into many more components than is possible in conventional one-dimensional electrophoresis. The first dimension is run in a tube gel (isoelectric focusing) which is then grafted horizontally onto the top of a polymerised slab gel. A small amount of agarose is used in the grafting process to bond the gels together. The second dimension is run on a vertical slab gel. After the run, the proteins appeared as spots in the slab.

Isoelectric focusing is a method in which the proteins are separated in a pH gradient according to their isoelectric points, that is the pH at which the net charge of the protein molecule is zero. At this pH, the protein molecules have no electrophoretic mobility. Isoelectric focusing is established in two stages. First, the pH gradient is formed. A mixture of highly mobile amphoteric compounds called ampholytes is mixed to the gel during its preparation. To generate the gradient, the current is turned on, and the ampholytes arrange themselves according to their own isoelectric points, the most acidic moving toward the anode, the most basic toward the cathode. In the second stage, the protein sample is layered on top of the gel, although it could be added in the first stage to the polymerisation mixture, before

establishment of the gradient. When a protein reaches its isoelectric point (pI) in the pH gradient, it carries a net charge of zero and will stop migrating. If it diffuses and moves away from its isoelectric point in the pH gradient, it will immediatly become charged and move back to its isoelectric pH and remain focused there. This gives a very high resolving power, in comparison with other separation methods where there is no force to counteract diffusion.

We have run SDS-3-16% gradient-PAGE electrophoresis with and without dithiothreitol as reducing agent used at 1 mM.

9- Dot blot: (Campbell, 1984)

We have used the dot blot assay based on the ELISA (Enzyme Linked Immuno Sorbent Assay) assay on nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad). ELISA is usually used for screening and quantitating an antibody.

In this assay, the antigen is bound to the plate or the membrane and incubated with the antibody which is piggy-backed by a second enzyme-linked antibody, with the enzyme driving a colometric reaction for detection . The second antibody could also be radioiodine labelled.

Most protein and nucleic acid antigens are passively adsorbed onto the solid surface by non specific hydrophobic interactions. The chemical nature of the solid support could be glass, nylon, sepharose ... or nitrocellulose paper. The plates or membranes are generally coated with material that encourages the binding of antigen. The capacity of the various supports vary widely and the amount of antigen adsorbed will depend on the

diffusion coefficient of the protein, the surface area to volume ratio and the time and temperature of the adsorption incubation. However, the binding is non covalent and in most systems, protein will leach off the plate during subsequent incubation with antibody, this can complex the antibody and reduce its binding. Consequently, there is no particular advantage to be gained from binding a large amount of antigen to the solid support and there may be disadvantages in binding too much. The incubation time with the antibody is balanced against the tendency of the antigen to leach off the plate during the incubation.

To avoid the adsorption of antibody by the support, once the antigen has been bound, it is usual to block any other sites (termed quenching) on the support using bovine serum albumin (1% w/v), gelatin (0.2-0.5% w/v), non-specific serum (1%v/v) or bovine γ-globulin (1% v/v) dissolved in phosphate buffered saline. The use of 0.01% Tween 20 or Triton X-100 in subsequent buffers also reduces the background as it discourages the formation of further hydrophobic interactions between the solid support and first and second antibodies. Additionally, if glutaraldehyde has been used, it is advisable to wash the plates or membrane with 100 mM glycine in the buffer to block off any remaining reactive glutaraldehyde sites.

The bound antibody is usually detected with a second antibody directed against it, usually against the total Ig G of the species in which the antibody has affinity. Covalently linked to this second antibody can be an enzyme with a chromogenic substrate. Sheep anti mouse or goat anti mouse enzyme-linked anti Ig G 's are available from a large number of commercial companies (Miles, Flow, Sigma, Bio-Rad, ..etc.)

Three types of enzymes could be used for color development with the second antibody. The earliest was alkaline phosphatase and currently horseradish peroxidase is commonly used. The third is β-galactosidase from E. coli which is active at the higher pH values preferred for antigen adsorption. Phosphatase and galactosidase are used in situations where there may be endogenous peroxidase activity in the antigen. The substrates generally employed for alkaline phosphatase and β-galactosidase are p-nitrophenylphosphate and o-nitrophenyl β-D-galactopyranoside respectively. In the case of horseradish peroxidase, several substrates are marketed although 0-phenylenediamine (OPD) is most commonly used.

Although it is usually possible to judge positive samples by eye, most ELISA are read in a spectrophotometer adapted for microtitre plates.

We have used the chequerboard ELISA as an initial screening test for determining the reactivity of the antisera. ELISA was used to determine the adequate antigen / antisera and antigen / antibody concentration, the signal to noise ratio (background) and some preliminary exploration of the density fractions (D_1-D_4). The system consisted of a 96-well microtitre plate with a constant concentration of antigen in every well, increasing serial dilution of antibody from column left to right, and repartition of the density fractions (D_1-D_4) by row. Control with no antigen occupies the last column, and control with no antibody occupies the last row.

10- Western blot: (Andrews, 1986)

A Western blot refers to the electrophoresis of the antigen followed by its subsequent transfer to nitrocellulose paper and incubation with specific antibody and with a labelled second antibody. The name originates from the use of Southern blots (Southern, 1975) for the analysis of DNA fragments on gels by labelled RNA probes and which consists of transferring DNA to nitrocellulose paper by a solvent flow procedure. When diazotized aminobenxyloxymethyl (DBM) paper was introduced, still employing solvent flow, the method became known as " Northern blotting". Inevitably, the transfer of proteins and nucleic acid to nitrocellulose was then known as "Western blot" (Towbin et al., 1979).

Western blot may be performed on conventional nitrocellulose matrix. The antigenic proteins will attach firmly but not covalently. Another alternative is the use of DBM paper which gives a covalent binding of the antigens, however DBM paper has limitations due to its extreme fragility.

The electrophoretic elution of the proteins or electroblotting consists of wetting a sheet of transfer membrane and placing it on the gel to be blotted which in turn is supported by a porous pad (e.g. 'Scotch Brite' scouring pads, foam sponge material or layers of wet blotting paper) mounted on a stiff plastic grid. To avoid trapping air bubbles between the layers, the manipulation could be done under transfer buffer. A second porous pad and plastic grid are then added above the transfer membrane, and rubber bands or a mechanical locking system are used to hold the grids together so that the gel and membrane are held tightly in close contact. The 'sandwich' is then placed in a tank of transfer buffer between two electrodes.

The transfer buffers used are of low ionic strength and buffers of many different compositions and pH have been used. Glycine and other constituents containing Amino groups react with diazotized aminobenxyloxymethyl (DBM) and diazophenylthioether (DPT) papers. Therefore, compounds containing glycine and Amino groups must not be present in transfer buffers with these papers. For the same reason, if glycine-containing buffer is used in the gels, glycine should be washed out of the gels with phosphate buffer before transfer. The incorporation of methanol increases the binding capacity of nitrocellulose for protein but it reduces the efficiency of protein elution from the gel, especially from SDS-PAGE gels. Long electroelution time, 12 hours or more, may be required for efficient transfer of large proteins. If methanol is omitted, gels may swell a little in low ionic strength buffers which can lead to band distortion on the blot. However, this can be overcome by presoaking the gel in transfer buffer for about 1 hour before transfer.

The transfer membrane must be on the correct side of the gel, so it is important to know the net charge on the molecules to be transferred. SDS-PAGE gels use approximately neutral or weak basic buffers and therefore the proteins migrate as anions and the nitrocellulose membrane should be on the anode side.

Immunological detection of proteins could be done through an ELISA assay as described in the dot blotting technique. Other traditional stainings (Amido Black, Coomassie Brilliant Blue, silver staining ...) have been used.

11- Coomassie blue staining:

Staining solution: 0.025% Coomassie Blue R250
10% acetic acid
25% isopropanol
overnight

Destaining solution: 10% methanol
10% acetic acid
overnight

Storage solution : 7% acetic acid

In this procedure, bands containing as little as 0.1µg of protein can be detected. The gels are soaked in the staining solution for 2-12h or overnight if more convenient.

Some small proteins may not be adequately fixed in the gel and can leached out during staining and destaining. If this is suspected, a prefixing step consisting of immersing the gel for 30 min in 10 % sulphosalicylic acid before staining should be included.

In the destaining procedure, we have found that adding a paper tissue greatly helps to absorb the dye.

12- Silver staining:

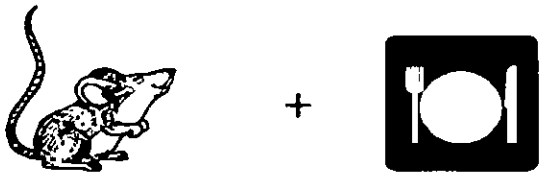
We have used the procedure described by Oakley et al., 1980.

This staining procedure is claimed to be 100 times more sensitive than the Coomassie Blue staining procedure, and extends staining methods down

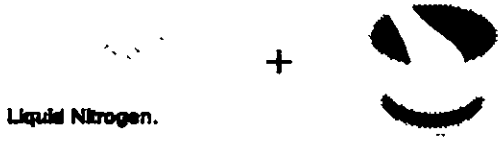
to the sensitivity levels of radioactivity procedures. A key factor for successful application appears to be removal of SDS prior to staining. Various washing regimes with solvent mixtures are used and as long as the washing is thorough and virtually all the SDS has been removed, the precise technique is clearly not critical. According to Oakley et al., with polyacrylamide gels of T > 10% the background may be unacceptably high and such gels should be prefixed in gel fix (50% methanol, 10% acetic acid, in glass distilled water) for at least 30 min followed by overnight washing in gel rinse (5% methanol, 7.5% acetic acid) before beginning the staining procedure.

CARTILAGE MATRIX EXTRACTION.

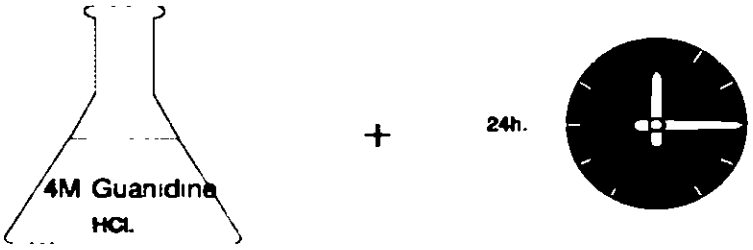
(1/3).



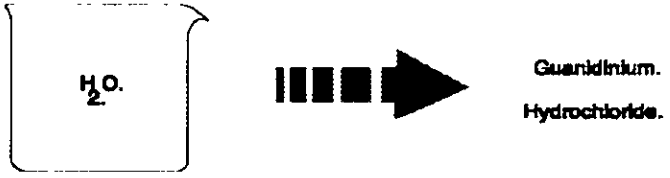
Surgical extraction, NS & CON.



Freezing and milling.



4M Guanidinium Hydrochloride Extraction

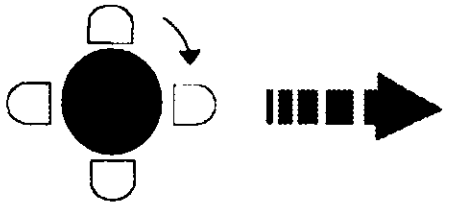


Dialysis against distilled demineralised water

To 2/3

CARTILAGE MATRIX EXTRACTION.

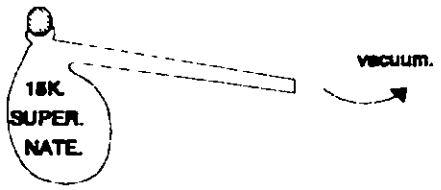
(2/3).



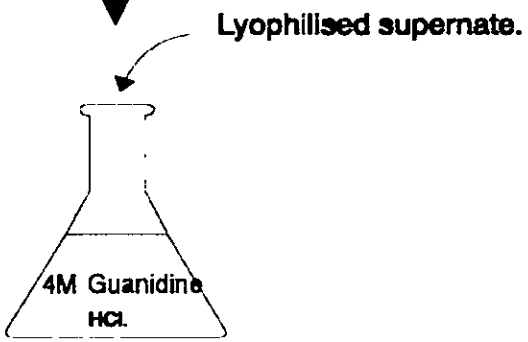
15K SUPERNATE.

15K PELLET.

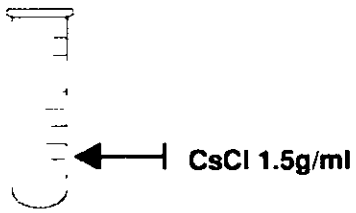
Centrifugation at 15,000rpm.



Lyophilisation of the 15K supernate.



Lyophilised supernate is dissolved in 12ml 4M Guanidine HCl

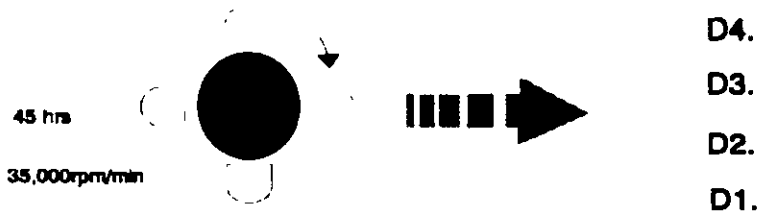


Cesium Chloride is added and adjusted. to 1.5g/ml.

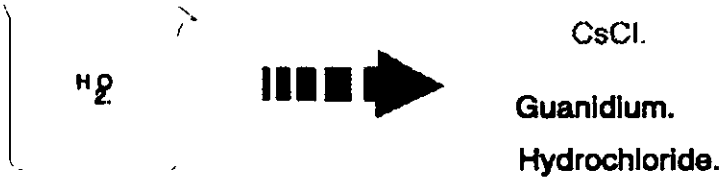
To 3/3

CARTILAGE MATRIX EXTRACTION.

(3/3).



CsCl gradient centrifugation.



Dialysis against distilled deionised. water.



Uronic acid and Bradford protein analysis

Dot Blot screening

1D 3-16% gradient SDS-PAGE and Western Blot

2D 3-16% gradient SDS-PAGE and Western Blot

EXPERIMENTS AND RESULTS.

Dot blot screening:

All the samples were used at full strength: 10µg of protein/100ml for D_3 and D_4 gradients, and10µg of uronic acid/ 100ml for D_1 and D_2 gradients. Then at 1/10, 1/100 and 1/1000 dilution. 300µl of samples were used for each well. 8A4 was utilized at 1/1000 dilution, and anti-PG diluted at 1/500.

Gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against monoclonal antibodies 8A4 and anti-PG (courtesy Dr. Mehbaran).

8A4 showed a positive reaction with nasal septum D_3 and D_4, and condyle D_3, from full strength until 1/10 dilution. These results indicated the presence of the link proteins in these fractions.

Anti-PG, specific for proteoglycans, did not show any positive reaction.

Chondroitinase ABC-pretreated gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against monoclonal antibody anti-PG. Only full strength samples were used.

The results confirmed that chondroitinase ABC pretreatment was necessary to expose the specific proteoglycan epitopes. Murine anti-human PG ascites reacted with nasal septum D_4, D_3, D_2, and D_1 fractions.

Interestingly D_4 and D_3 fractions were more reactive than D_2, and D_1 fractions for condyle.

We found that the nasal septum proteoglycans in the 11-day rat are located in all the gradient fractions (D_1 through D_4) of the nasal septum cartilage, and that they are more abundant in the gradient fractions D_4 and D_3 than D_2 and D_1 of the condyle cartilage.

Chondroitinase ABC-pretreated gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against Rabbit Anti Rat Nasal Septum antisera (RARNS) and Rabbit Anti Rat Condyle antisera (RARC) (Hans, 1981) respectively. Samples were tested at full strength, 1/10 and 1/100 dilution.

Raw antisera were tested at full strength for reactivity with the different gradient fractions. These antisera did show positive reactivity with their respective targets. Nasal septum reacted stronger with D_4, D_3, and D_1 than with D_2 from full strength to 1/100 dilution. Condyle showed more reactivity with D_4 than D_3 and only at sample full strength, reactivity with D_2 and D_1 were not higher than the background. For nasal septum, sensitivity was down to 1/100 dilution, while for condyle it did not pass 1/10 dilution.

Gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against Rabbit Anti Rat Nasal Septum antisera (RARNS) and Rabbit Anti Rat Condyle antisera (RARC) respectively. Samples were tested at full strength, 1/10 and 1/100 dilution.

This experiment is the same as the above, but **none** of the samples were treated with chondroitinase ABC. This experiment also confirmed that predigestion with chondroitinase ABC is required to expose the antigenic proteoglycan epitopes. Reactivity was weaker at D_1 and D_2 than at D_3 and D_4 . For nasal septum, sensitivity was down to 1/100 dilution, while for condyle it did not pass 1/10 dilution.

Chondroitinase ABC-pretreated gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against Rabbit Anti Rat Condyle antisera (RARC) and Rabbit Anti Rat Nasal Septum antisera (RARNS) respectively. Samples were tested at full strength, 1/10 and 1/100 dilution.

This experiment tests the cross-reactivity of the full strength antisera between nasal septum and condyle. Cross-reactivity exists between nasal septum and condyle antisera and sensitivity is down to 1/10 dilution of the sample. In particular, the nasal septum gradient fractions exhibited positive reactivity at all gradient levels, while condyle samples showed more reactivity at D_4, D_3, and D_1 than D_2 . The resulting cross-reactivity is substantial especially when the nasal septum fractions were reacted against rabbit anti rat condyle antisera (RARC).

Chondroitinase ABC-pretreated gradient samples (D_1 to D_4) of 11-day rat nasal septum and condyle cartilage were blotted against

Rabbit pre-immune sera. Samples were tested at full strength and 1/10 dilution.

This experiment was designed to test the background noise and assessed the signal to noise ratio. Condyle full strength D_4 sample did react very lightly. Accounting the pre-above dot blots, the background noise (Pre-immune sera, Chondroitinase ABC) does yield some coloration, enough to make 1/100 dilution for the sample unreadable or negative. Thus, substantial amounts of material would be saved if dot blotting in the future were limited to full strength to1/10 dilution of the samples.

Full strength and 1/10 dilution samples of different gradient levels from nasal septum and condyle were assayed against Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) used at full strength, 1/10, 1/100/ 1/500, and 1/1000 dilutions.

This test was designed to assess the sensitivity of the cartilage samples versus the ANS-IgG and vice versa. The D_2 and D_1 fractions were chondroitinase ABC-predigested. Assuming that the proteoglycans are located mostly in D_2 and D_1 fractions, the enzyme predigestion would allow the epitopes to be uncovered.

The ANS-IgG reacted with the nasal septum samples at D_1 which indicates reactivity mostly with the proteoglycans, and at D_3 and D_4 with the matrix proteins and proteoglycans. Some cross-reactivity was seen with the condyle samples at D_1, D_2, and D_4 which confirmed the cross-reactivity test done with the raw antisera. The ideal concentration antigen versus antibody

appeared to be full strength to 1/10 dilution of sample versus 1/100 dilution of ANS-IgG.

Full strength and 1/10 dilution samples of different gradient levels from nasal septum and condyle were assayed against Rabbit Anti Rat Condyle IgG (AC-IgG) used at full strength, 1/10, 1/100/ 1/500, and 1/1000 dilutions.

This assay showed that AC-IgG is not very specific for condyle as cross-reactivity with nasal septum samples was substantial at all gradient fractions. This would again emphasize that condyle and nasal septum do share many common antigens as was found above with the cross-reactivity assay of raw antisera. The practical concentration for dot blot would be full strength to 1/10 of cartilage sample versus 1/10 to 1/100 dilution of AC-IgG.

Staining of 1D electrophoresis:

Samples of different gradient levels from nasal septum and condyle were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then stained with Coomassie Blue. Chondroitinase ABC predigested sample of D_1 and D_2 gradients from both nasal septum and condyle were included in the run. Sample concentration of 10μg of protein/well was used.

Based on our dot blot results, it was decided to start the1D SDS-PAGE with 10µg of protein/well (full strength in dot blot) as sample concentration.

Track A received sample buffer and was quite blank.

Track B (the rainbow standard marker) showed regular bands, from top to bottom, and expressed the apparent molecular weight in kilodaltons: 200, 97.4, 69, 46, 30, 21.5, 14.3, 6.5 kD.

Track C, with Chondroitinase ABC (C_{ABC}) alone as control, stained bands can be attributed to the proteins present in the C_{ABC} preparation. discrete and diffuse bands above and below 200 kd, a dark band around 97.4 kd, a dark thick band around 66 kd (most likely bovine serum albumin used in the enzyme preparation) other discreet bands above and below 46kd.

Track D, nasal septum D_1 gradient non-C_{ABC} predigested was virtually blank.

Track E, nasal septum D_1 gradient C_{ABC} predigested: in the high molecular size above 200 kd appeared two adjacent bands, most likely L_I and L_{II} large core proteins of the proteoglycans which presented an apparent molecular weigh of about 300,000-450.000kD (Sommarin et al. (1986)), the width of the bands reflected the polydispersity of the proteoglycans. The band pattern belonging to the proteins present in the preparation of C_{ABC} was superimposed here as the samples were C_{ABC} predigested. Right below 46 kD there was a light band, S_2, that seemed to correspond to the core protein of small proteoglycans (Heinehard and al., 1981), liberated by C_{ABC} digestion. The protein background of C_{ABC} could be removed by chromatography on a column of Sepharose CL-4B as described by Oike et al., 1982.

Track F, nasal septum D_2 gradient non-C_{ABC} predigested: a broad feeble smear extended above 200 kD, a very discreet band showed at 69 kD, another broad medium dark smear bridged 46 to 30 kD, and several individual bands appeared between 21 and 6 5 kD. The latter are proteins other than proteoglycan as they were unchanged in track G where the same sample was C_{ABC} predigested.

Track G, nasal septum D_2 gradient C_{ABC} predigested: enzyme digestion allowed the bulky proteoglycan to penetrate the gel by severing the glycosaminoglycans, a heavy core protein was located discreetly above 200 kd, at the same level as heavy core proteins in D_1. As in D_1 but much more prominent, a small core protein, S_2, appeared below 46 kD. A broad smear above 30 kD and stained bands between 21 and 6.5 kD were believed to be non-proteoglycans proteins for reasons cited above.

Track H and I, nasal septum D_3 and D_4 gradient non-C_{ABC} predigested: no core protein of proteoglycan was seen here, but a prominent band of matrix protein was seen between 97 4 and 200 kD. The rest of the matrix protein was scattered as visible sharp bands below this prominent band in D_3, and below and above this band in D_4 . One of the bands around 45 kD in both tracks might be the link protein.

Track J, sample buffer, virtually blank.

Track K, condyle D_1 gradient C_{ABC} predigested: much more reduced appearance of large core protein was found here than in the nasal septum where only L_I was seen. Light core protein S_2 was not spotted.

Track L, condyle D_2 gradient non-C_{ABC} predigested, showed a 69-kD smear that did not exist in the nasal septum. The 46-to-30-kD smear was

equivalent to the nasal septum, but slightly different in quality though, with two central and adjacent bands. The quality and the quality of the proteins scattered below 30 kD were obviouly different from those in the nasal septum.

Track M, condyle D_2 gradient C_{ABC} predigested A few more bands were spotted and they responded to the pattern of the proteins involved in C_{ABC} preparation (Track C) No large core protein appeared as in the nasal septum. The quality and the quantity of the proteins bands below 30 kd were similar to the non-digested sample and different from the nasal septum.

Track N, condyle D_3 gradient. Track O, condyle D_4 gradient. They both showed prominent bands, between 200 and 97 4 kD, and 46 and 30 kD, and scattered bands below 30 kd which differed from the nasal septum in both quality and in quantity.

Samples of different gradient levels from nasal septum and condyle were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then stained with silver staining. Chondroitinase ABC predigested sample of $D_1$and D_2 gradients from both nasal septum and condyle were included in the run. Sample concentration of 1μg of protein/well was used.

This gel confirmed the findings shown above with Coomassie Blue, however it opened prospects for screening with minute amount of protein. 1 μ g instead of 10 μg or more usually used with Coomassie Blue

Western Blot of 1D SDS-PAGE electrophoresis:

Samples of different gradient levels from nasal septum were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with monoclonal antibody 8A4 (1/1000), specific for link protein. $D_1$and D_2 gradient samples were Chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

This gel shows a positive reactivity especially in the matrix fraction (D_3 and D_4). D_4 contains a high number of link protein aggregates (46 kD) and fragments (97, 30, 21 and 14 kD) which are normally found in high turnover tissues like cartilage matrix. These aggrgates could be formed during the dissociative extraction procedure. An increasing concentration gradient of link protein is apparent from D_1 to D_4 , which is consistent with the previous conclusion found with dot blotting.

Samples of different gradient levels from nasal septum were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with monoclonal antibody 2B6 (1/500), specific for chondroitin-4-sulfate. $D_1$and D_2 gradient samples were Chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

The positive reactivity shows presence of Chondroitin-4-sulfate in abundance in the large core protein of D_1 (> 200 kD), which is very subtle at the same level of D_2 . Positive reactivity was found at S_2 (< 46 kD, small core protein), and less intense in D_1 than in D_2 . The reactivity with 2B6

after cleavage with chondroitinase ABC suggests that these core proteins are Chondroitin-4-sulfate proteoglycans. No reactivity was found in D_3 and D_4 .

Samples of different gradient levels from nasal septum were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with monoclonal antibody 3B3 (1/5000), specific for chondroitin-6-sulfate. $D_1$and D_2 gradient samples were Chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

Broad dark bands of 3B3 positive material in D_1 extending largely above 200 kd showed that the nasal septum contains a lot of chondroitin-6-sulfate. Positive reactivity also identified two core protein fragments (≤ 200 kd, and ≥ 96 kD). In D_2, only a light reactivity appeared as one of the large core proteoglycans. There was no reactivity in D_3 . D_4 was not C_{ABC}-treated and revealed a 3B3 minus epitope having approximately the apparent molecular weight of the large core proteoglycans. D_1 and D_2 are routinely treated by chondroitinase ABC before SDS-PAGE electrophoresis in order to remove the glycosaminoglycans that would have impeded the aggregate to enter the gel. Three other positive bands were identified in the D_4 fraction, extending from 69 to 30 kD, they might be proteoglycan fractions or matrix proteins.

Samples of different gradient levels from nasal septum were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with monoclonal antibody 5D4 (1/10,000), specific for

keratan sulfate. $D_1$and D_2 gradient samples were chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

Positive reactivity appeared in the high molecular weight core protein in both D_1 and D_2. No reactivity was observed in D_3 , as was seen with 3B3. D_4 shows a positive broad band, same as with 3B3, and indicates that this core protein containing 5D4 epitope is likely the same as the one with 3B3 minus epitope. D_4 also revealed three scattered positive bands in the range 69-30 kD. Superimposition of these patterns on Coomassie staining or on the 3B3 experiment above would allow discrimination between the proteoglycans fractions and matrix proteins.

D_1 and D_2 gradient samples from nasal septum were chondroitinase ABC and keratanase predigested then subjected to 1D 3-16% gradient SDS-PAGE electrophoresis and Western blotted with Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) at 1/100 dilution. Sample concentration of 10μg of protein/well was used.

D_1 shows that the antisera contains an antibody that reacts with the core protein (300-450 kD) and a fragment in the 69 kD which might be associated with the hyaluronate binding domain (70-80 kD, Heinegard et al., 1987). D_2 does not show any reactive epitope.

Samples of different gradient levels from nasal septum and condyle were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with rabbit preimmune sera. $D_1$and D_2 gradient samples were chondroitinase ABC predigested .

Sample concentration of 10μg of protein/well was used.

This experiment afford the opportunity to check the background. There was no reactivity.

Samples of different gradient levels from nasal septum and condyle were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) at 1/100 dilution. D_1 and D_2 gradient samples were chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

This blot shows that D_1 and D_2 needed to be C_{ABC}-treated in order to give positive reactivity. In the nasal septum, substantial reactivity exists at all level of D_4 which appeared rich in antigenic material, at all gradient level the matrix proteins between 46 and 21 kD were reactive, and in D_1 the large core proteoglycans were also reactive. Cross-reactivity with the condyle was apparent and confirmed the dot blot findings.

Samples of different gradient levels from nasal septum and condyle were subjected to 1D 3-16% gradient SDS-PAGE electrophoresis, then Western blotted with Rabbit Anti Rat Condyle IgG (AC-IgG) at 1/50 dilution. D_1 and D_2 gradient samples were chondroitinase ABC predigested . Sample concentration of 10μg of protein/well was used.

There was more reactivity in the nasal septum than in the condyle, with substantial cross reactivity. In addition the anti rat condyle Ig G was not

very specific to the condyle. The salient reactive sites in the nasal septum are in the D_1 large core proteoglycans and througout the matrix protein of D_4 .

Staining of 2D electrophoresis:

Only 20μg of protein sample was needed for silver staining.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then silver stained. 20μg of protein sample was used.

The gel shows superior resolution of low molecular weight proteins. There is a typical pattern of the binding region fragments and the link protein (marked). A group of proteins appeared below 46 kD, they are still undefined.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then Coomassie blue stained. 50μg of protein sample was used.

Staining was very faint, it would be interesting to increase the sample concentration two to three-fold for the next experiment.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then silver stained. 50μg of protein sample was used.

There was some better separation than without reducer.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then silver stained. 20μg of protein sample was used.

This blot shows an abundance of 97-66-47-30 kD matrix proteins in D_4 of nasal septum, some of them are undefined. The next logical attempt would be to use 2D blot to locate the protein reactive with the antisera.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then silver stained. 20μg of protein sample was used.

There was much better resolution than without dithiothreitol.

2D Western blot:

Our testing revealed that 50μg of protein sample is the minimum to obtain readable reaction. The concentration of antibody was the same as for 1D Western blot.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with rabbit preimmune sera(1/10). 50μg of protein sample was used.

There was no reaction.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis with 1mM dithiothreitol, then Western blotted with rabbit preimmune sera(1/10). 50μg of protein sample was used.

No reaction was seen.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with monoclonal antibody 8A4 (1/1000). 50μg of protein sample was used.

Link protein appeared around and under 46 kD. In addition several fragments exist as seen in 1D gel. No reaction with the matrix proteins under 46 kD was spotted in the 2D silver staining.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis with 1mM dithiothreitol, then Western blotted with monoclonal antibody 8A4 (1/1000). 50μg of protein sample was used.

Microheterogenity of link protein is very well demonstrated here. Several streaks were seen parallel to each other, and were well defined after cleavage of the disulfide bonds by the reducer.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) at 1/100 dilution. 50 μg of protein sample was used.

There was reactivity with link protein and with the proteoglycan binding fragments. There was no antibody against the undefined matrix proteins that were seen in the silver staining.

Nasal septum gradient D_3 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then Western blotted with Rabbit Anti Rat Nasal Septum IgG(ANS-IgG) at 1/100 dilution. 50μg of protein sample was used.

Same reactivity pattern as on the blot without dithiothreitol, but in addition some resolution of the microheterogenity of link protein was shown.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with rabbit preimmune sera (1/10). 50μg of protein sample was used.

No positive background reaction was seen.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then Western blotted with rabbit preimmune sera (1/10). 50μg of protein sample was used.

No background reactivity was seen.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with monoclonal antibody 8A4 (1/1000). 50µg of protein sample was used.

This blot reflects what was seen on 1D blot with link protein appearing under 2D as streaks at 97, 46, 30 kdD and under.

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then Western blotted with monoclonal antibody 8A4 (1/1000). 50µg of protein sample was used.

Microheterogeneity of link protein is better visualised here than with the Western blot without reducer

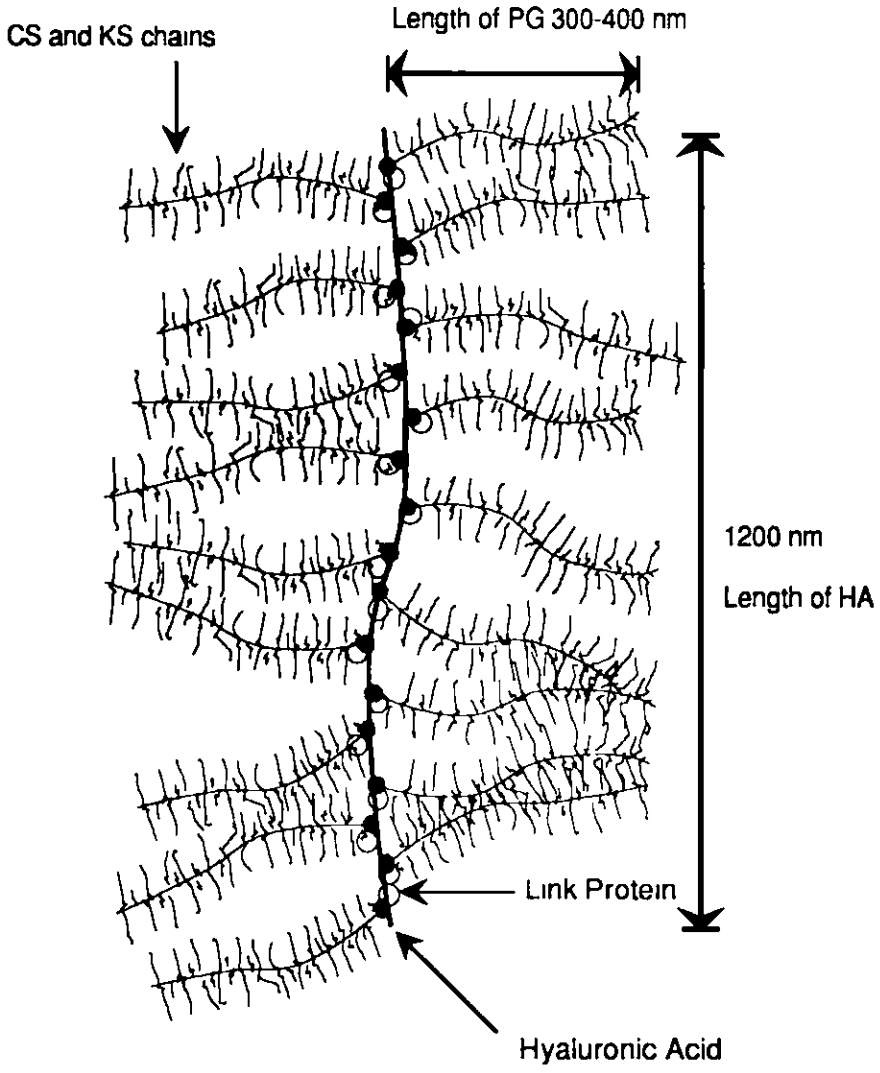
Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis under non-reducing condition, then Western blotted with Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) at 1/100 dilution. 50µg of protein sample was used.

Streaks of positive reactivity with link protein were seen above and below 46 kd. Some undefined matrix proteins spotted in the silver stained gel showed positive reactivity with ANS-IgG, they are localised with their pI, Mr: (6.9-7, 49-59), (6.85-6.92, 37), (6.4, 54), (6.2-6.3, 53), (6.1, 53), (5.9, 51), (5.9-6, 46).

Nasal septum gradient D_4 was subjected to 2D SDS-PAGE electrophoresis with 1 mM dithiothreitol, then Western blotted with

Rabbit Anti Rat Nasal Septum IgG (ANS-IgG) at 1/100 dilution. 50μg of protein sample was used.

The reducer did improve the resolution of link protein without enhancing the separation of the undefined proteins.

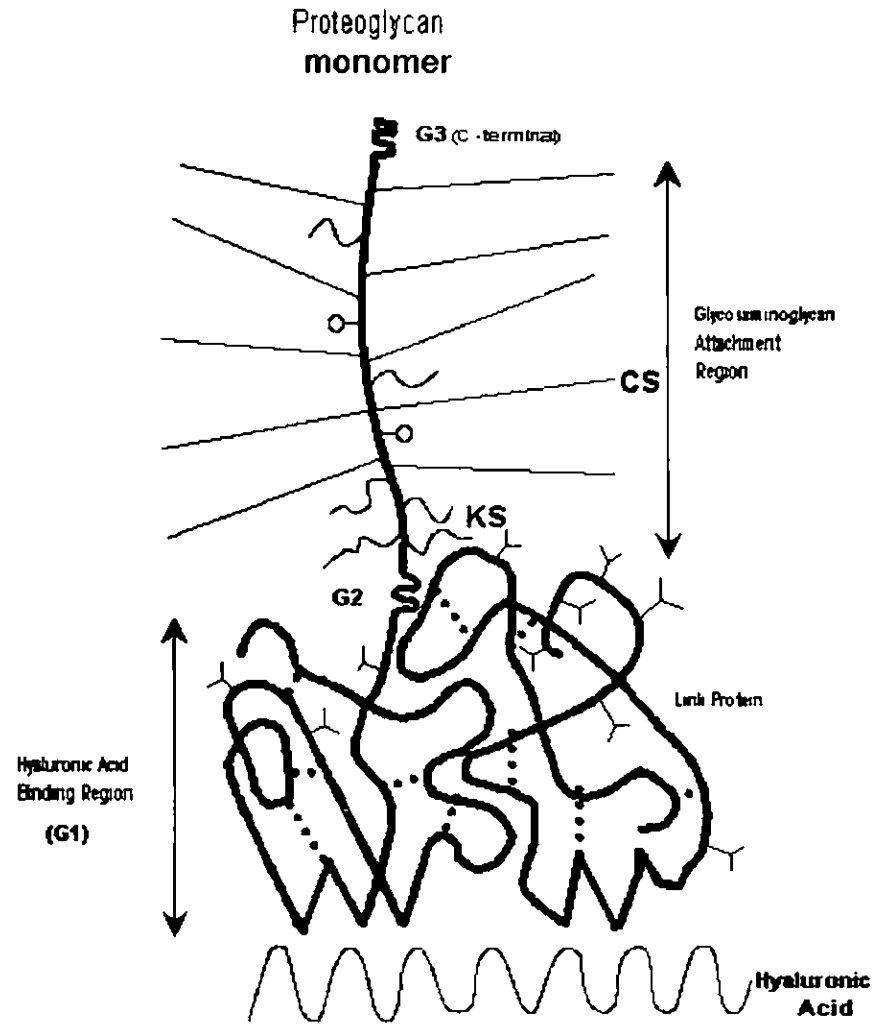


Model of proteoglycan aggregate from mature cartilage (Muir, 1980)

CS, chondroitin sulfate; KS, keratan sulfate; PG, proteoglycan.

HA, hyaluronic acid.

○ link protein ● hyaluronate binding domain



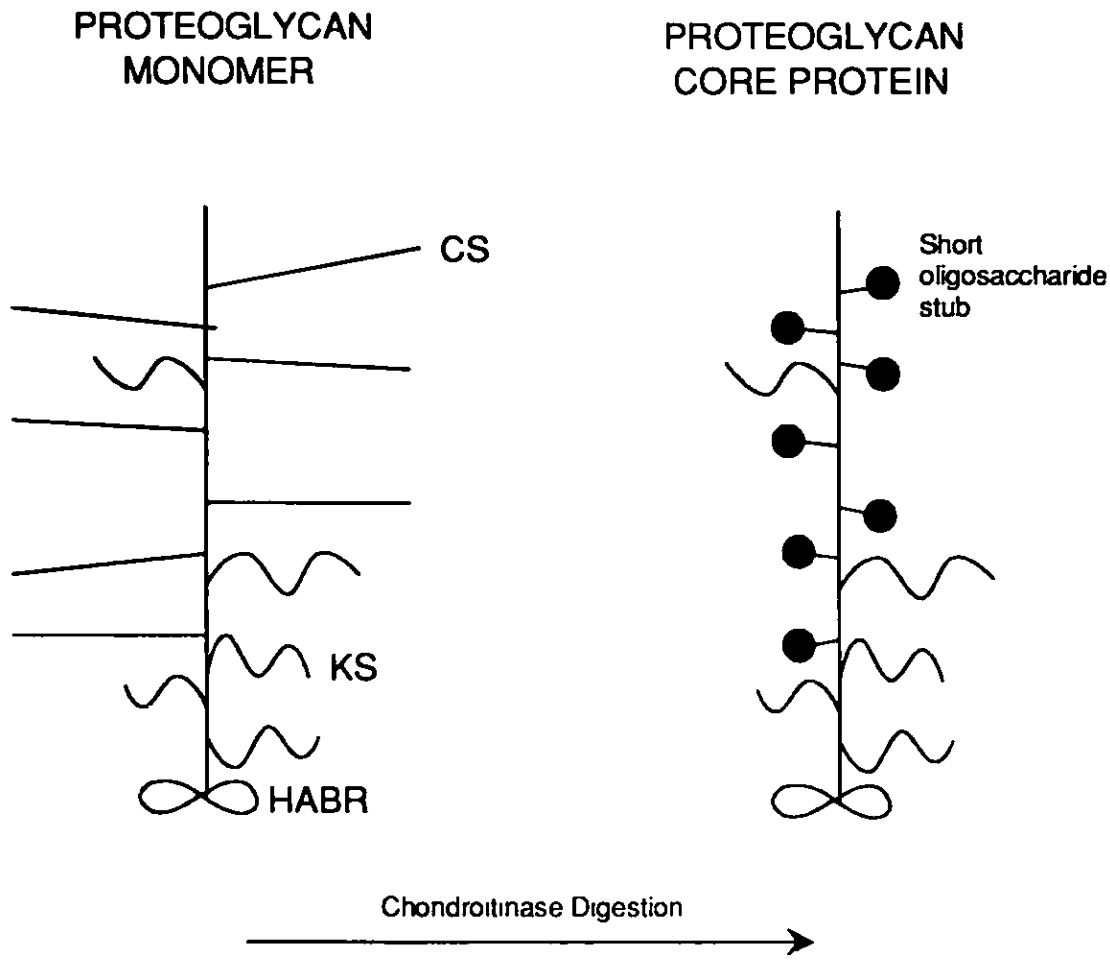
Schematic model of cartilage proteoglycan aggregate substructures

. N-linked oligosaccharides O-linked oligosaccharides

CS chondroitin sulfate **KS** keratan sulfate

G1,2,3 globular domains

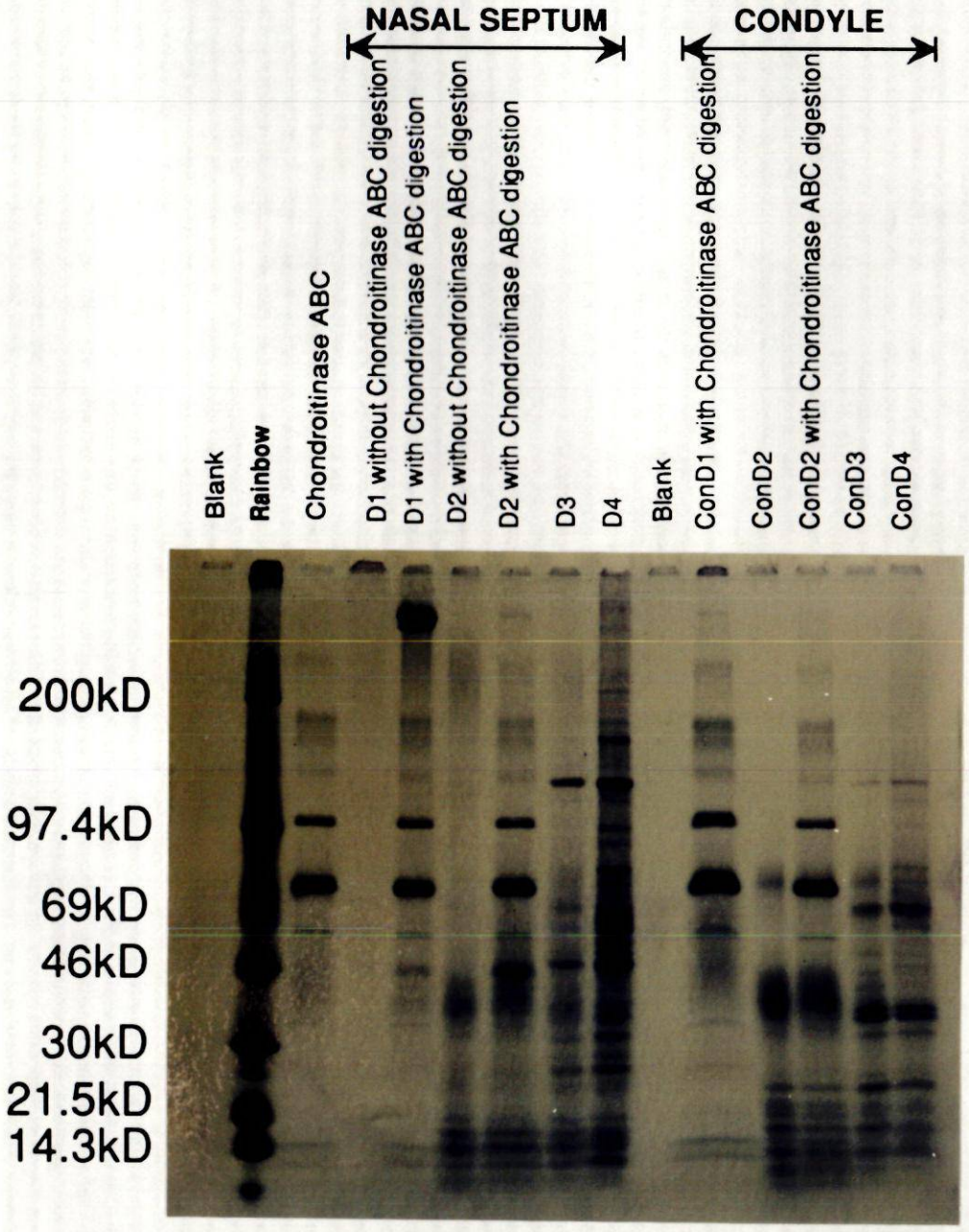
(Modified from Caterson et al 1981)



Schematic representation of the characteristic oligosaccharide stubs of chondroitin sulfate that result from chondroitinase digestion of cartilage proteoglycan monomer This allows the core protein to enter the gel. CS. chondroitin sulfate. **KS**: keratan sulfate **HABR**: hyaluronic acid binding region.

mAb	Ig Isotype	Reactive Site
5-D-4	$IgG_1(\kappa)^b$	Recognizes an epitope present in the disaccharide structure common to both skeletal and corneal keratan sulfate.
3-B-3	$IgM(\kappa)$	Recognizes delta-unsaturated or saturated tetrasaccharides derived from chondroitin-6-sulfate but not chondroitin-4-sulfate. These sites can be generated by either chondroitinase or testicular hyaluronidase digestion of chondroitin sulfate proteoglycans. Reactivity with non-enzyme digested proteoglycan suggest presence of atypical terminal chondroitin-6-sulfate.
8-A-4	$IgG_{2b}(\kappa)$	Recognizes a chondroitin-6-sulfate epitope common to link protein isolated from proteoglycan aggregate from a wide variety of animal species (i.e., rat, bovine, human, and chicken hyaline cartilages). This determinant is present on all subpopulations of the link protein(s) i.e., link protein 1, 3, or 3 as observed after SDS-polyacrylamide gel electrophoresis of the link protein.
2B6	IgG_1	Anti-chondroitin-4-sulfate. Requires predigestion with chondroitinase to generate its epitope consisting of a non reducing terminal delta 4,5 unsaturated uronic acid residue adjacent to N-acetylgalactosamine-4-sulfate. Both chondroitinase ABC and ACII generate this epitope in chondroitin sulfate glycosaminoglycan chains, but only chondroitinase ABC generates the epitope from dermatan sulfate glycosaminoglycans. Differential enzyme digestion with chondroitinase ABC or ACII can be used to distinguish the occurence of chondroitin sulfate and dermatan sulfate in samples.

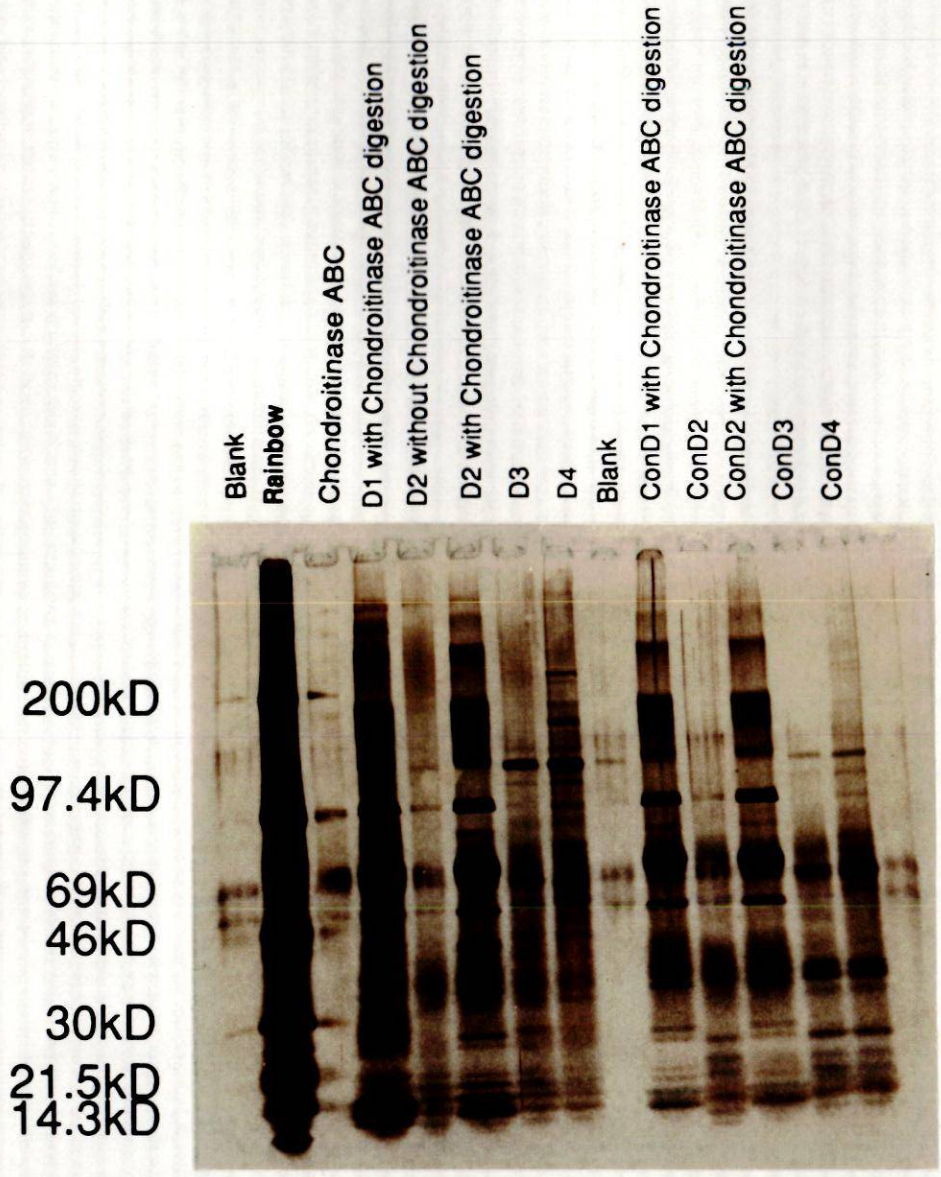
Caterson et al. (1985, 1990)



Coomassie Blue Staining, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum and condyle cartilage samples were both used at 10 micrograms/well.

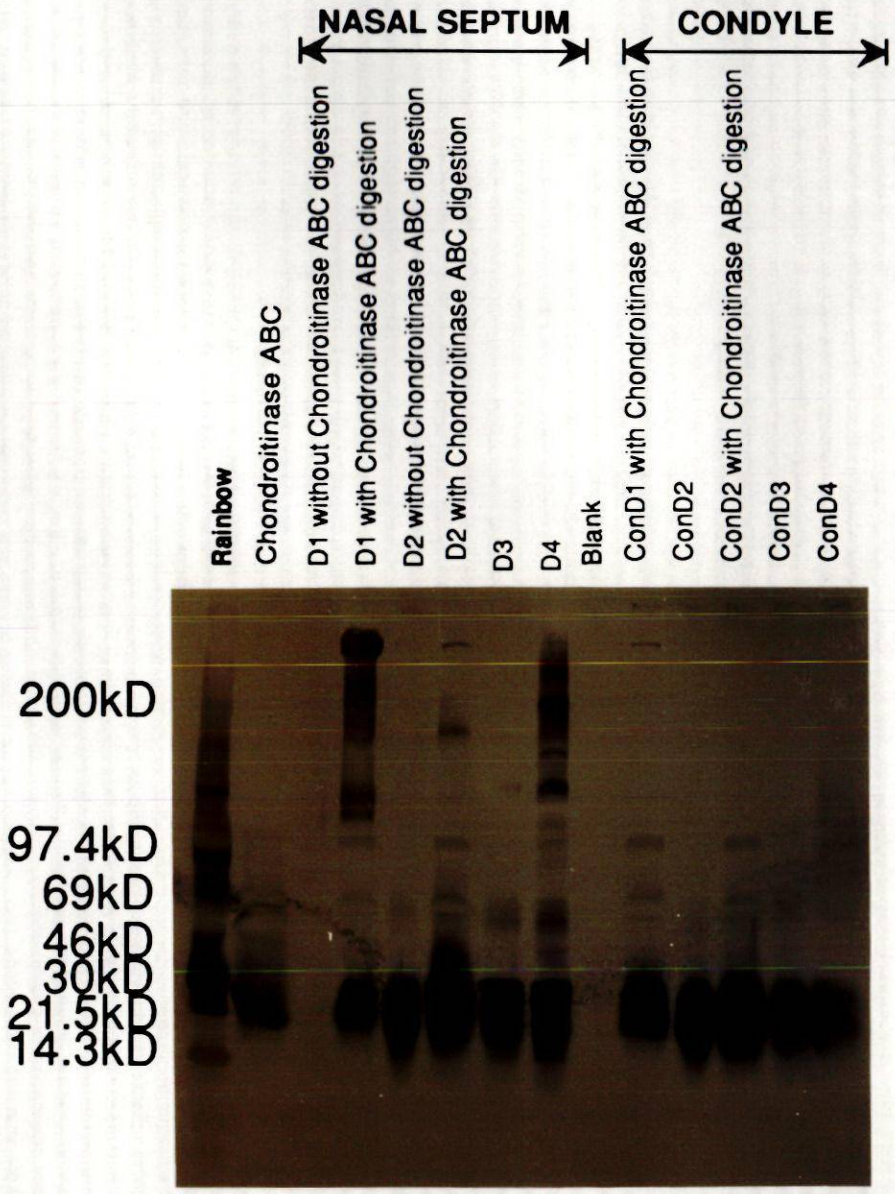
Differences in quality and quantity exist between Nasal septum and Condyle.



Silver Staining, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum and condyle cartilage samples were both used at 1 microgram /well.

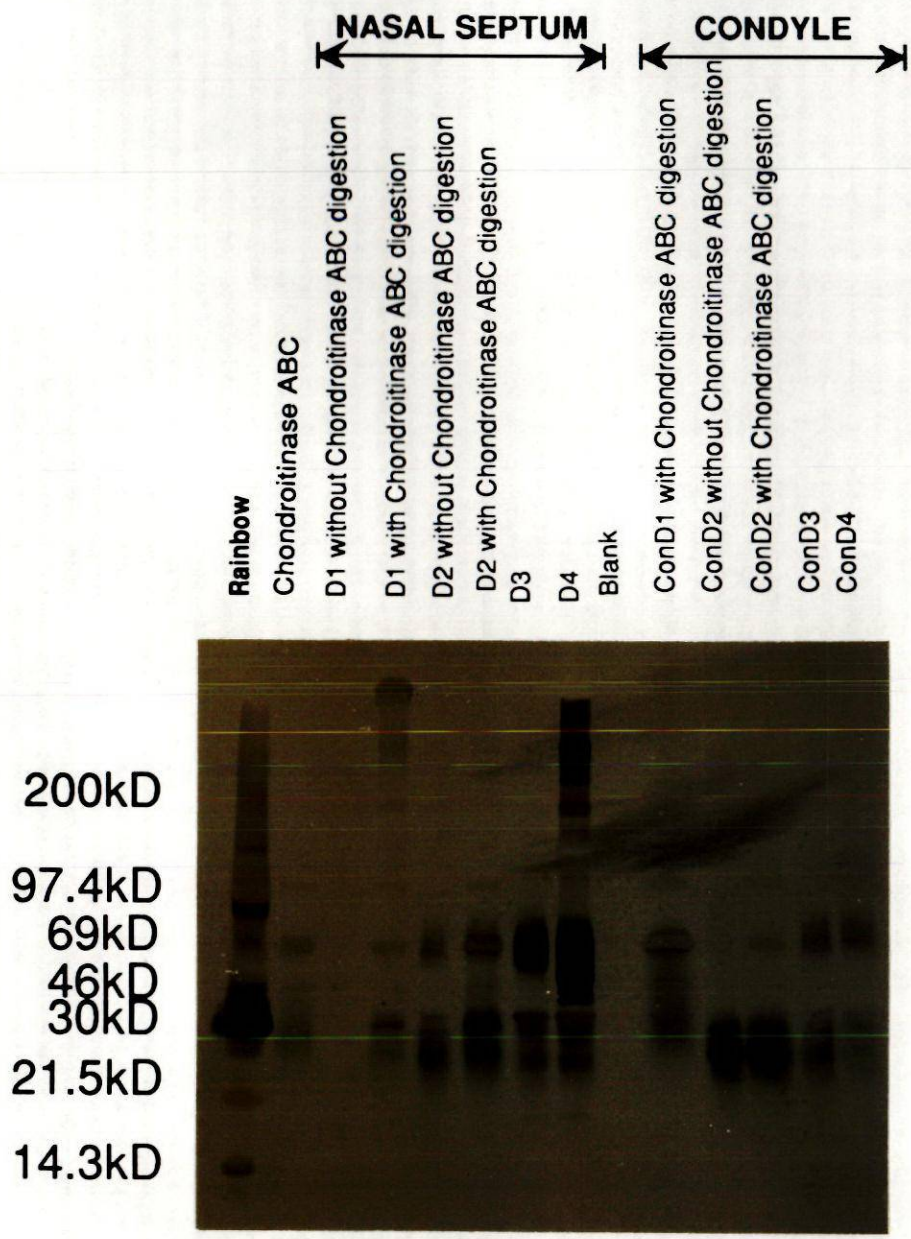
Differences in quality and quantity exist between Nasal Septum and Condyle.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum and condyle samples were used at 10micrograms of protein/well, Anti Rat Condyle IgG at 1/50 dilution.

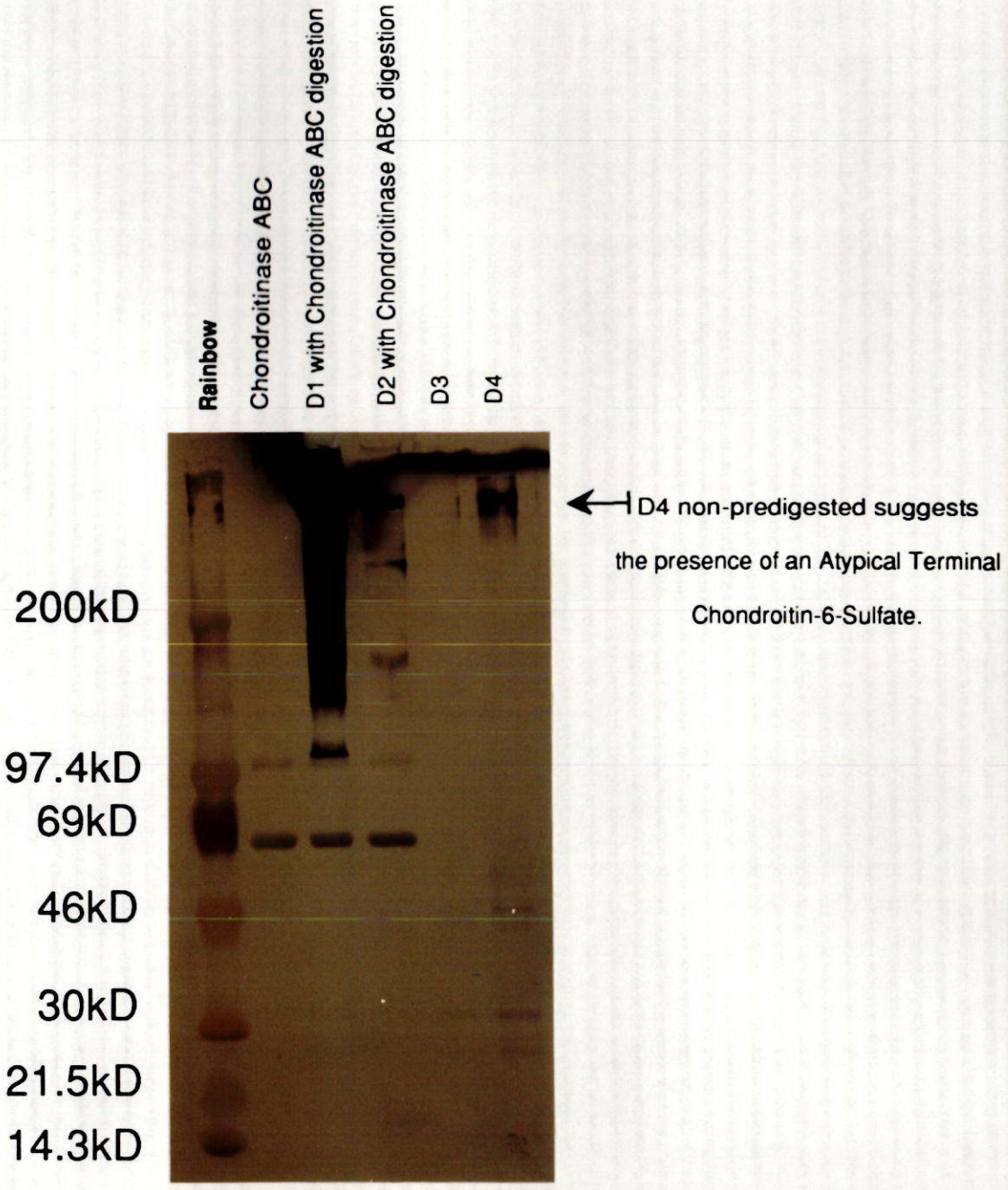
Cross reactivity is substantial, Anti Rat Condyle IgG is not very specific to the Condyle.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum and condyle samples were used at 10micrograms protein/well, Anti Rat Nasal Septum IgG at 1/100 dilution.

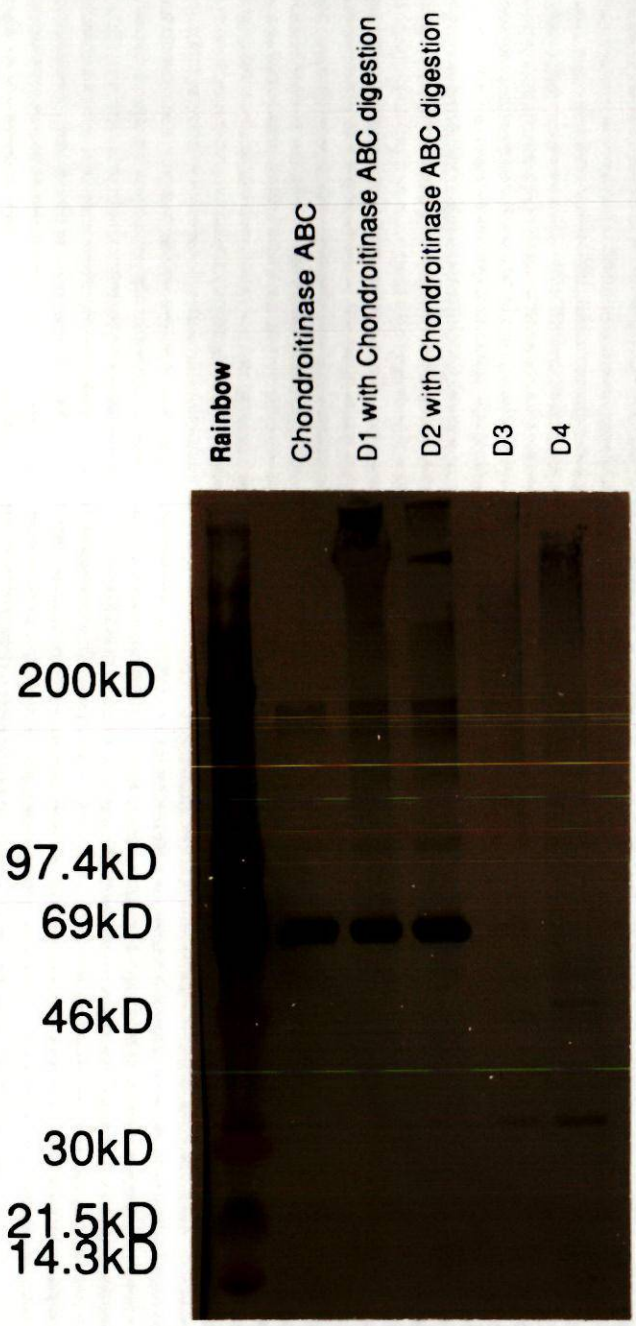
Cross reactivity with the condyle is apparent. D4 of the nasal septum shows substantial reactivity at all molecular weight level.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum samples were used at 10micrograms of protein/well, mAB 3B3 at 1/5000 dilution.

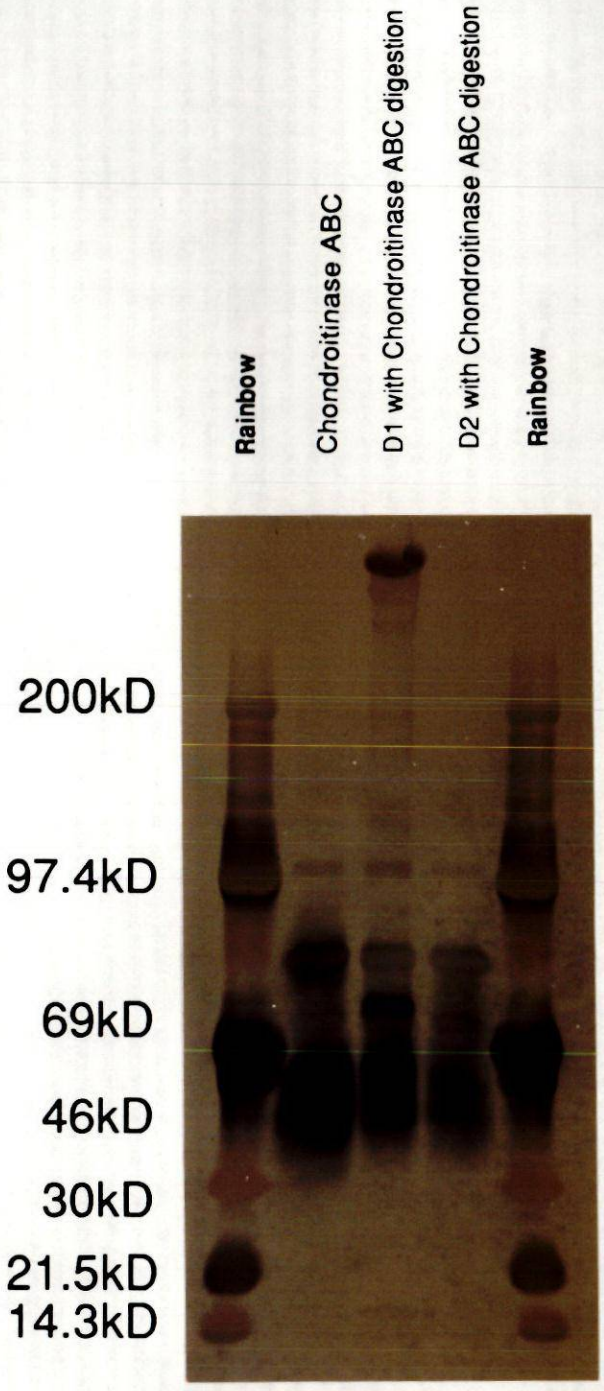
D4, non Chondroitinase ABC treated, suggests the existence of a subset of Chondroitin-6-sulfate proteoglycans.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum samples were used at 10micrograms of protein/well,
mAb 5D4 at 1/10,000 dilution.

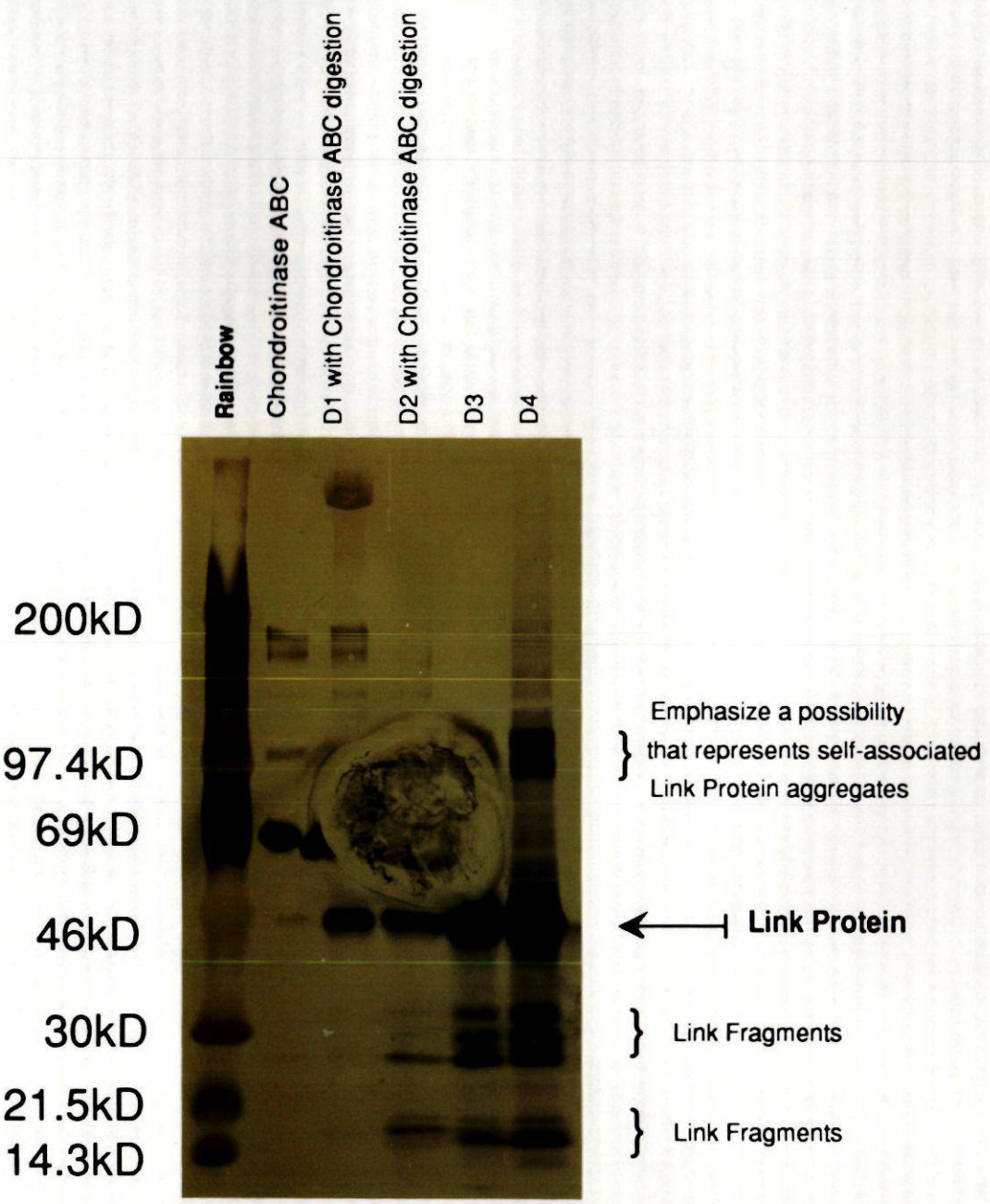
Rat proteoglycans react with mAb 5D4. Superimposition with Coomassie Blue would allow to discriminate the proteoglycans fractions from matrix proteins.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum samples (10micrograms protein/well) were Chondroitinase ABC and Keratanase digested. Anti Nasal Septum IgG was used at 1/100 dilution.

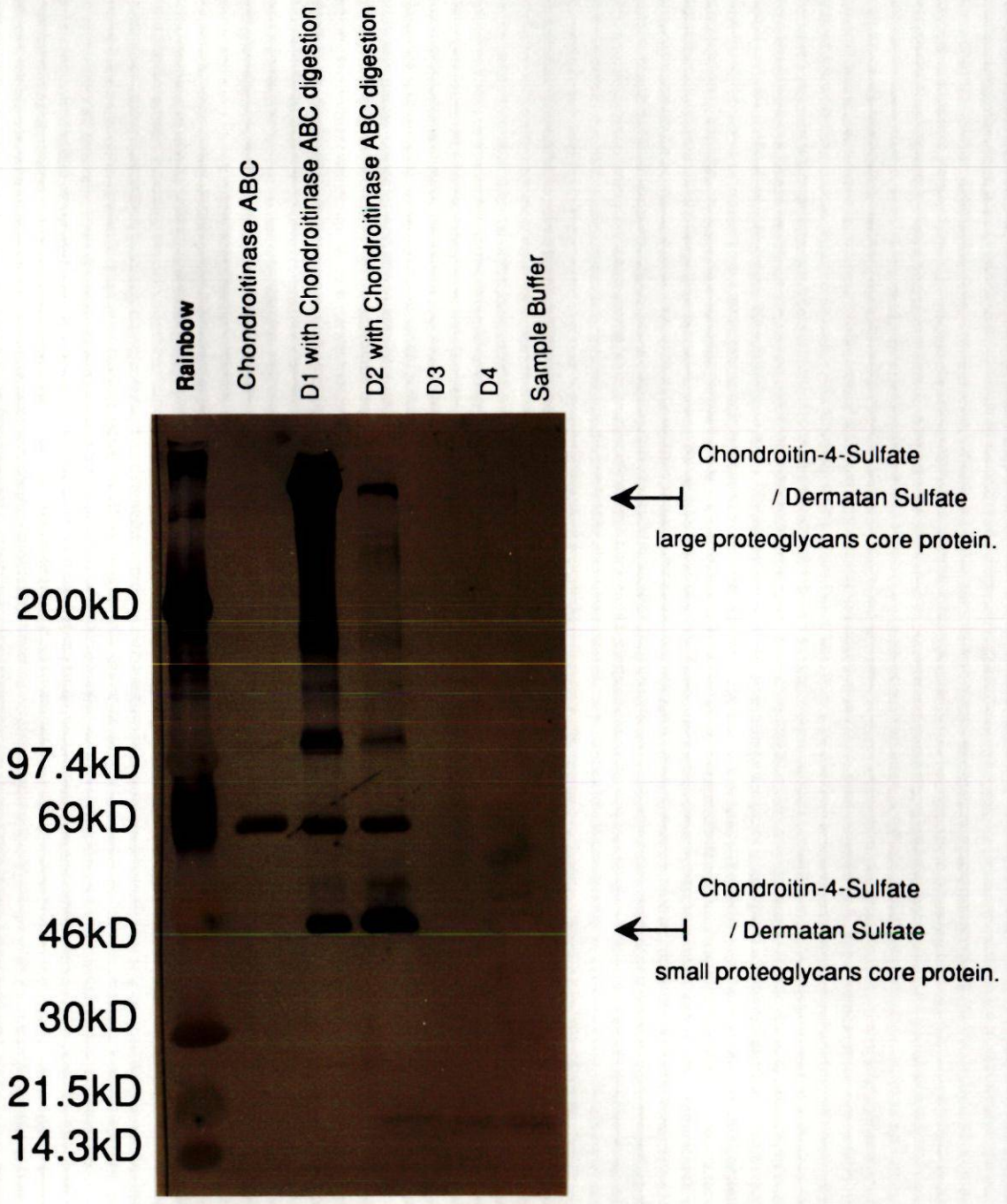
The antisera contains an antibody that reacts with the heavy core protein (300-450kD) and a fragment in the 69kD which might be associated with the hyaluronate binding domain.



Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum samples were used at 10micrograms protein/well. mAb 8A4 at 1/1000 dilution.

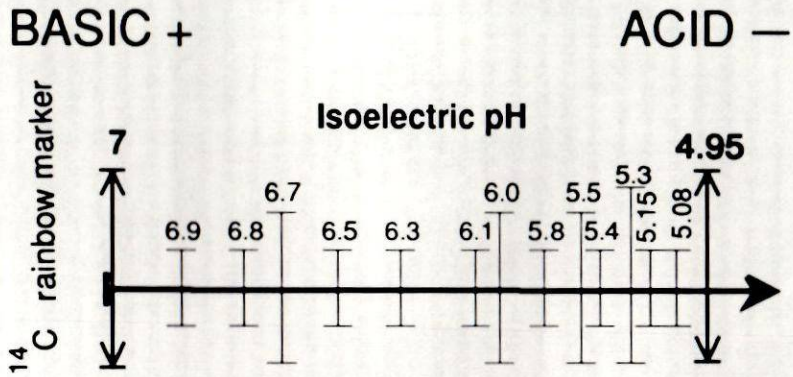
Increasing concentration gradient of Link Protein is apparent from D1 to D4. Link aggregates and fragments are normally found in high turnover tissues like cartilage matrix.

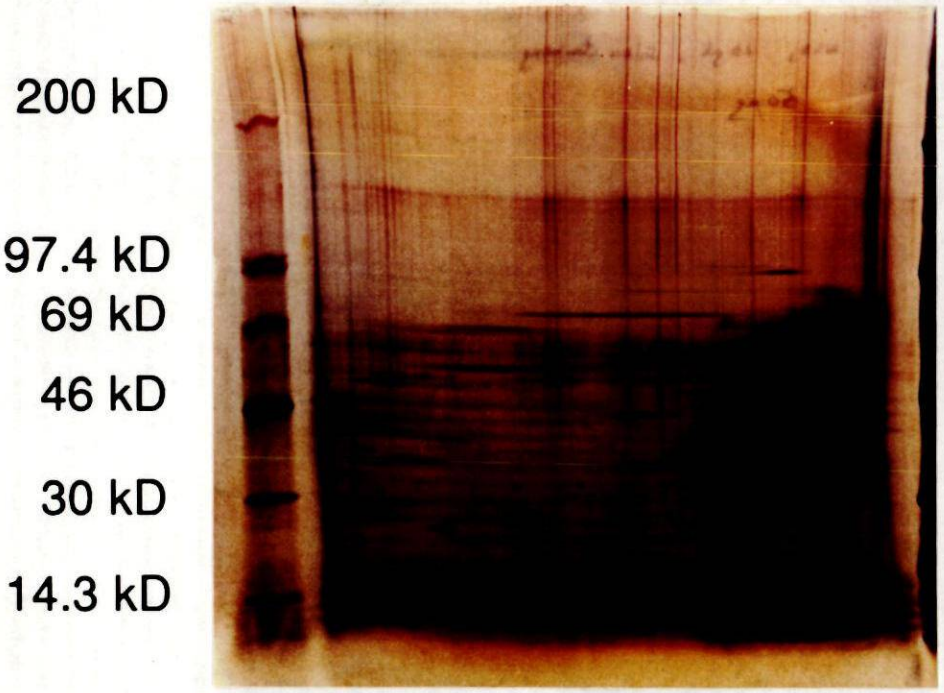


Western Blot, 1D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum cartilage sample were used at 10micrograms protein/well. mAB 2B6 at 1/500.

Positive reactivity shows presence of Chondroitin-4-Sulfate / Dermatan Sulfate
D3 and D4 show no reactivity.





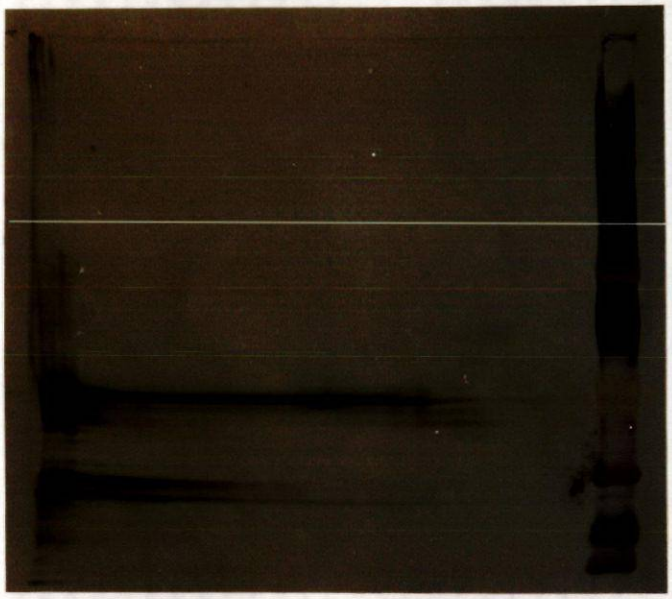
Silver Staining, 2D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum cartilage D3 sample was used at 50 micrograms of protein.

Undefined proteins appear below 46kD.

BASIC +

ACID —



200 kD

97.4 kD

69 kD

46 kD

30 kD

21.5 kD

14.3 kD

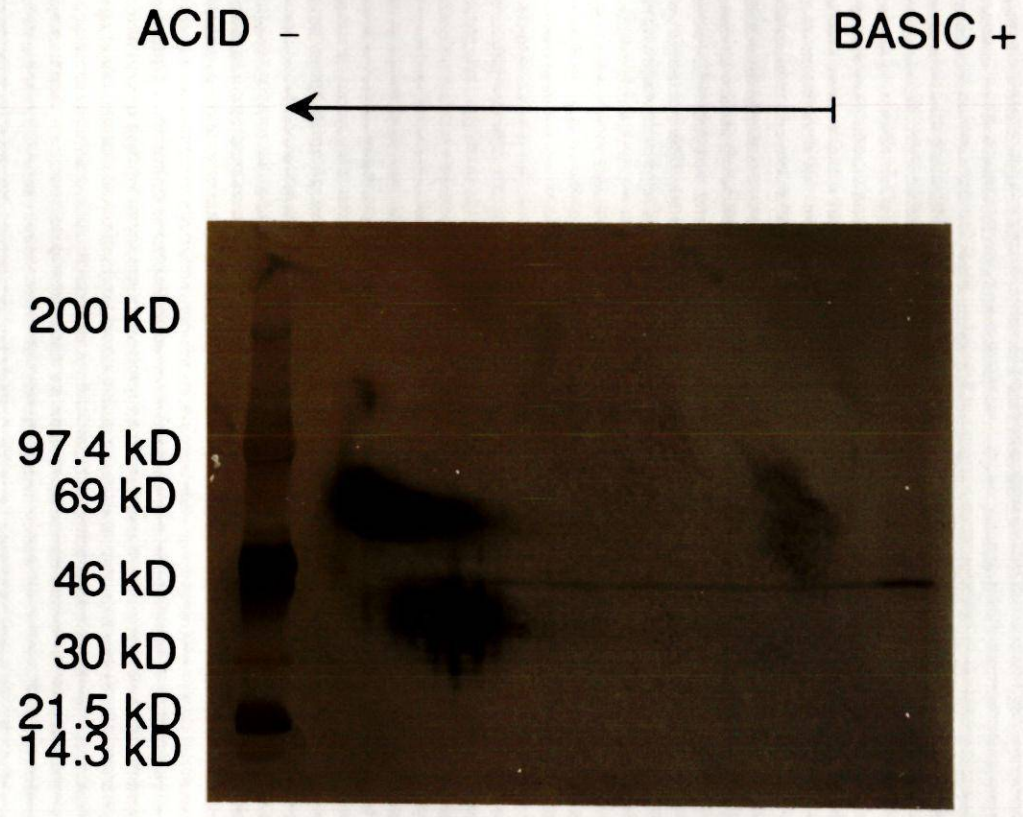
Link Protein
and Microheterogeneity

Link Fragments
and Microheterogeneity

Western Blot, 2D 3-16% SDS-PAGE Electrophoresis

Nasal septum sample D3 was used at 50 micrograms of protein. mAb 8A4 at 1/1000.

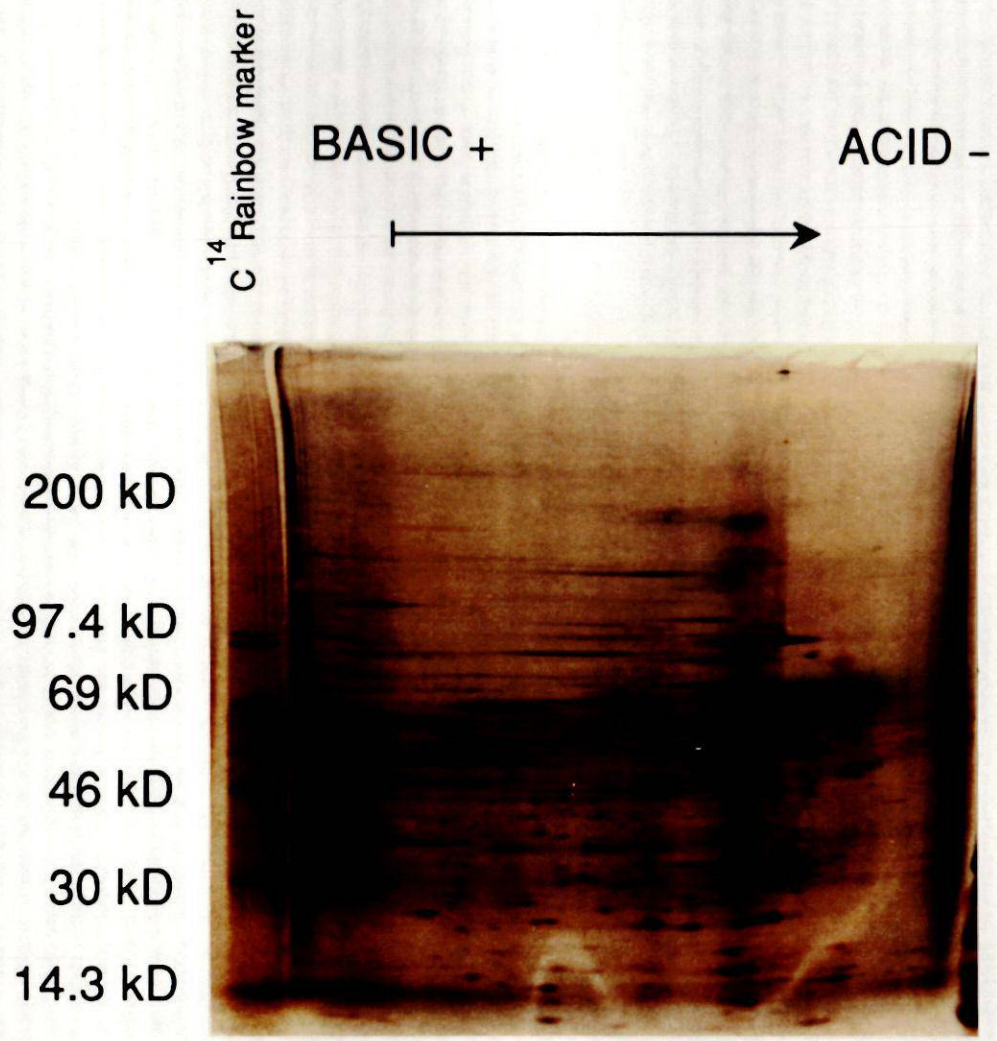
Positive reactivity shows Link protein. There is no reactivity with the matrix proteins below 46 kD.



Western Blot, 2D 3-16% SDS-PAGE Electrophoresis

Nasal septum sample D3 was used at 50 micrograms of protein. Anti NS IgG at 1/100.

There was no reactivity with the undefined matrix proteins below 46 kD.



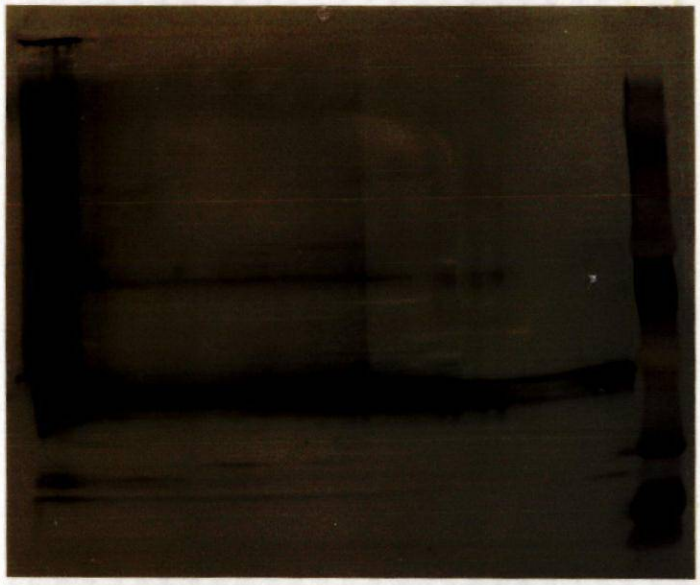
Silver Staining, 2D 3-16% SDS-PAGE Electrophoresis

Nasal septum sample D4 was used at 20micrograms of protein.

There is an abundance of matrix proteins from 30 to 97.4kD.

BASIC +

ACID −



200 kD

97.4 kD

69 kD

46 kD

30 kD

14.3 kD

Link Protein
and Microheterogeneity

Link Fragments
and Microheterogeneity

Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum sample D4 was used at 50micrograms of protein. mAb 8A4 at 1/1000.

Link protein is revealed at 97.4, 46, 30kD and under.

BASIC +

ACID –

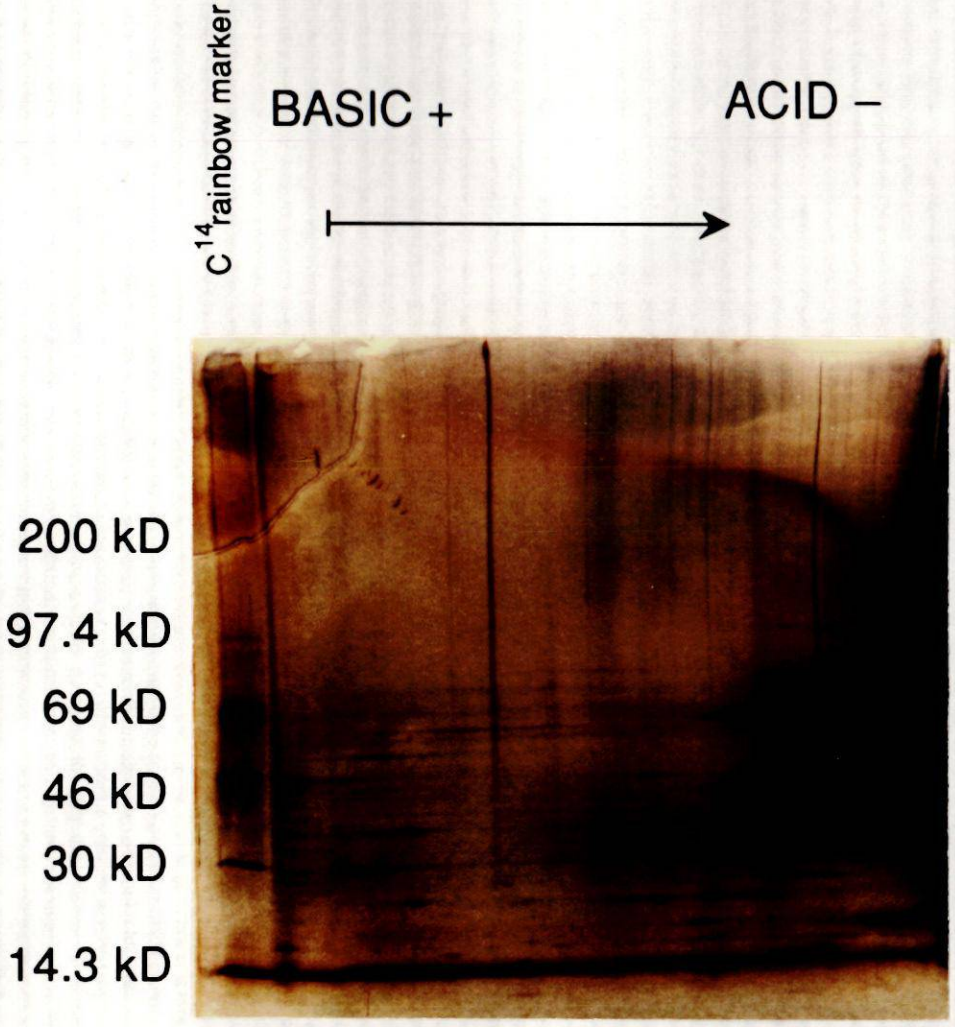


Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis

Nasal septum sample D4 was used at 50micrograms of protein. Anti NS IgG at 1/100.

Some undefined matrix proteins showed positive reactivity with Anti NS IgG.

They are yet to be defined.

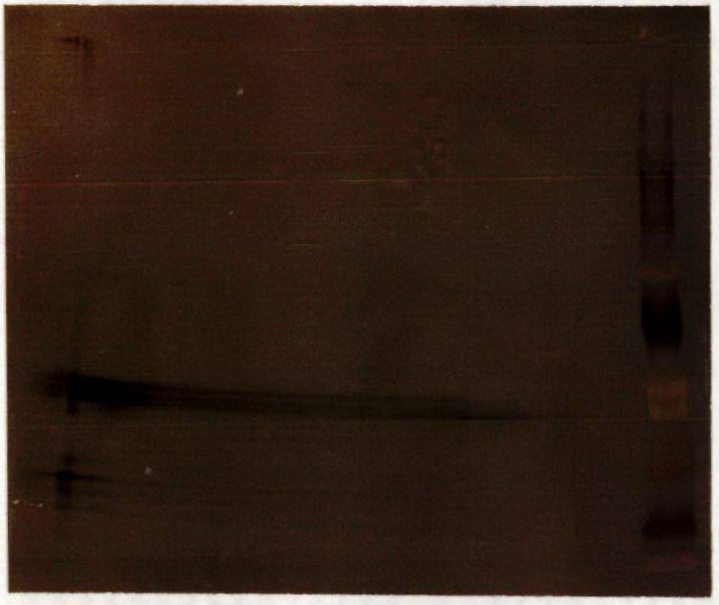


Silver Staining, 2D 3-16% gradient SDS-PAGE Electrophoresis with 1mM Dithiothreitol

Nasal septum sample D3 was used at 50micrograms of protein.

BASIC +

ACID –



200 kD

97.4 kD

69 kD

46 kD

30 kD

21.5 kD

14.3 kD

Link Protein
and Microheterogeneity

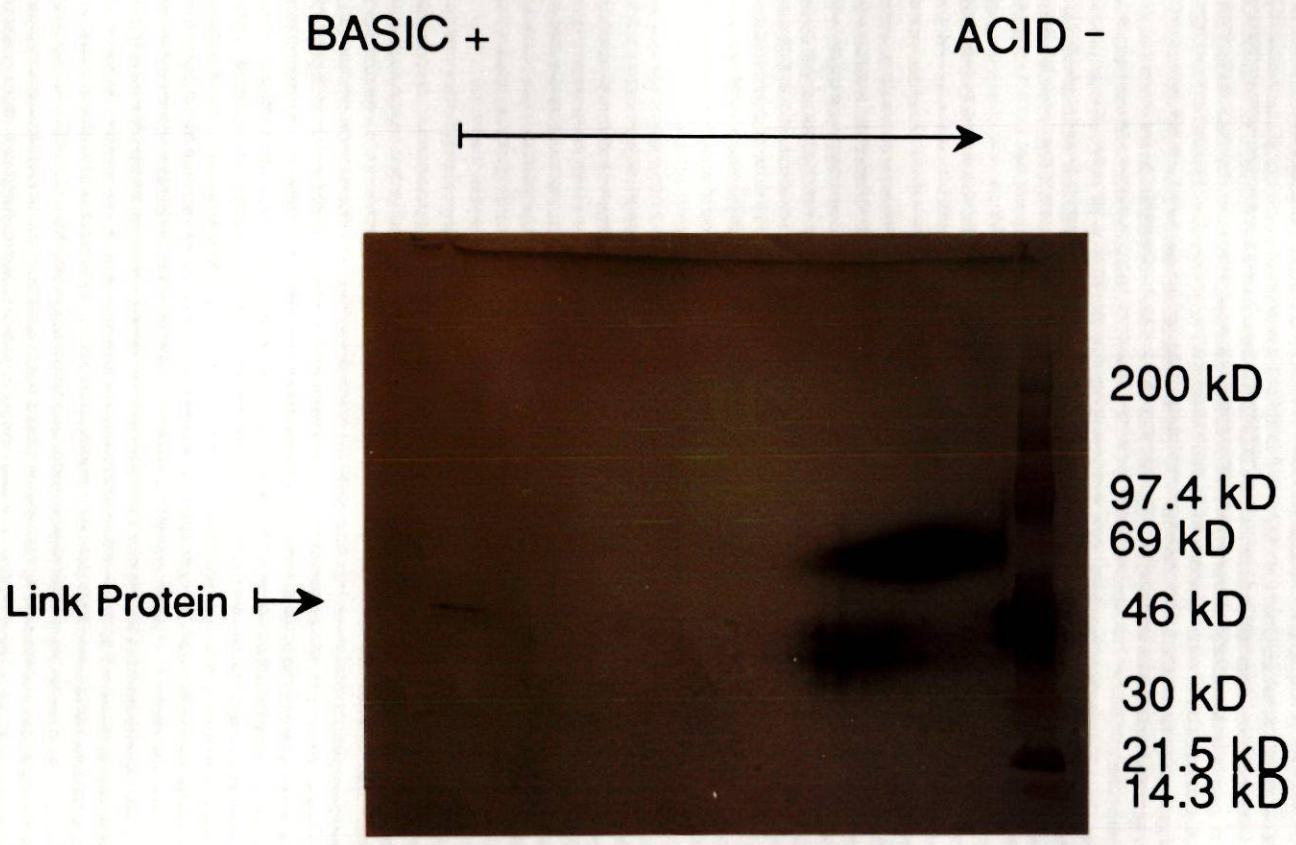
Link Fragments
and Microheterogeneity

Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis

with 1mM Dithiotreitol

Nasal septum sample D3 was used at 50micrograms of protein. mAb 8A4 at 1/1000.

Clear evidence of microheterogeneity of Link protein.



Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis.

with 1mM Dithiothreitol

Nasal septum sample D3 was used at 50micrograms of protein. Anti NS IgG at 1/100.

There was no reactivity with the undefined matrix proteins below 46kD.

BASIC+ ACID -



200 kD

97.4 kD

69 kD

46 kD

30 kD

21.5 kD

14.3 kD

Link Protein
and Microheterogeneity

Link Fragments
and Microheterogeneity

Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis

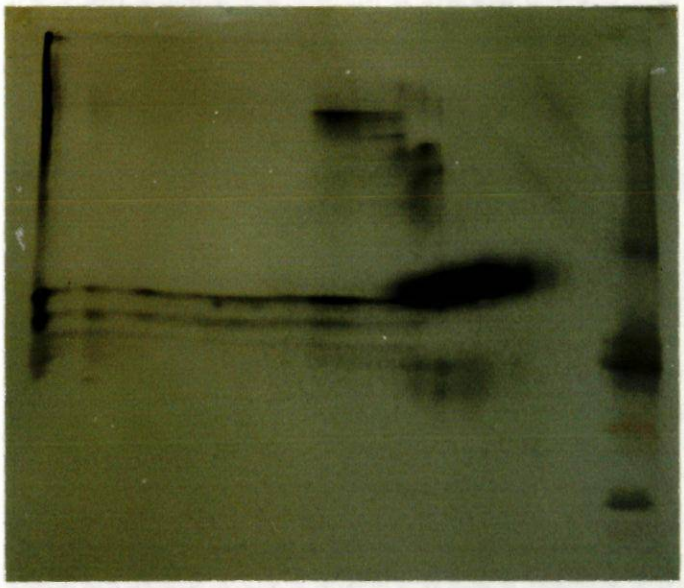
with 1mM Dithiotreitol

Nasal septum sample D4 was used at 50micrograms of protein. m Ab 8A4 at 1/1000.

Clear evidence of microheterogeneity of Link protein.

BASIC +

ACID −



200 kD
97.4 kD
69 kD

46 kD

30 kD

21.5 kD
14.3 kD

Link Protein
and Microheterogeneity

Western Blot, 2D 3-16% gradient SDS-PAGE Electrophoresis

with 1mM Dithiothreitol

Nasal septum sample D4 was used at 50micrograms of protein. Anti NS IgG at 1/100.

The reducer improved the resolution of Link protein without enhancing the separation of the undefined reactive proteins.

DISCUSSION

Slodov (1991) demonstrated that proteins unique to the cartilage found in the condyle, nasal septum and epiphyseal plate cartilages could most likely be isolated from the extracellular protein of whole tissue rather than from the matrix or cell membrane of cultured rat chondrocytes. Hans (1981) showed that injection of anti-rat nasal septum antisera into growing rats altered midfacial growth. The identification of specific matrix protein components, especially those interacting with the polyclonal antisera used by Hans, would be the first step in understanding the biomechanical control mechanism that is involved in facial development .

This study showed that the majority of the antigenic matrix proteins are located in the D_4 and D_3 CsCl gradient fractions rather than D_2 and D_1. In condylar cartilage the fraction D_4 contains more antigenic proteins than the D_3. These results were evidenced by dot blotting nasal septum and cartilage samples with their respective raw antisera. Antigenic potential exists in the dense core proteins of the proteoglycans (300-450 kD) in D_1 of the nasal septum and throughout the matrix fractions for both condyle and nasal septum as pointed out on one dimensional Western blots. Most of the dot blot reactivity tests with the nasal septum were sensitive to a higher sample dilution than with the condyle. This suggests that either there is a higher concentration of antigenic material in the nasal septum matrix fraction compared to the condyle or that the anti-nasal septum antisera are much more reactive as they were both used at full strength and with comparative

sample dilutions. The first hypothesis is more likely and it was confirmed by the results of the SDS-PAGE gels stained with Coomassie Blue showing that particularly D_3 and D_4 from the nasal septum are much richer in matrix proteins than those from the condyle. The same gel under silver staining confirms the results found with Coomassie Blue staining, with the advantage of consuming less sample, 1μg versus 10μg, but requiring much more manipulation. The condyle represents an overall appearance of protein pattern similar to the nasal septum, however differences in quantity versus quality were evidenced by a precise observation, and as stated, the nasal septum matrix fractions were more reactive and richer in antigenic material. These findings led us to focus our investigation on the nasal septum for the rest of the study.

The heavy core protein in D_1 fraction (300-450 kD) of the nasal septum was also reactive with Rabbit Anti Rat Nasal Septum Ig G's (ANS-IgG). This was demonstrated by Western blot with ANS-IgG after successive digestion with chondroitinase ABC and keratenase to remove the side chains. This same core protein in D_1 also contains keratan sulfate (5D4 positive), chondroitin-6-sulfate (3B3 positive), and chondroitin-4-sulfate/dermatan sulfate (2B6 positive after C_{ABC} digestion). Other smaller fragments in the 69 KD range were also reactive. They appeared to correspond to the hyaluronate binding domain, and the presence of these fragments is the normal product of high turnover tissues like the nasal septum of growing rat studied here. Thus, these core molecules belonging to the D_1 fraction of growing rat nasal septum are typical proteoglycans reported in the literature.

The D_2 and D_3 fractions of the nasal septum were not reactive with ANS-IgG when subjected to 1D and 2D electrophoresis. In D_3, silver staining of 2D gel pointed out a group of undefined matrix proteins widely spread below 46 kD, however neither 8A4, nor ANS-IgG reacted with these matrix proteins. It may be that the antisera against these matrix proteins were already adsorbed, or these proteins were not present or not present in sufficient concentration in the cartilage extract when inoculated into the rabbits. One possible explanation of the absence of reactivity of these matrix proteins in D_3 is that it might be due to the fact that the electrophoresis was carried out in gels and buffers containing SDS or urea and these denature the antigens which may not react subsequently with the antibodies. The antigen might have renatured during the transfer to nitrocellulose paper for Western blot, and it was suggested to remove SDS from the membrane bound proteins by simple washing or by a further brief electrophoresis in the tranfer apparatus with SDS-free buffer (Andrews, 1986).

In D_4 of the nasal septum, silver staining of the 1D gel revealed an abundance of matrix protein in the range of 97-66-47-30 kD. Reactivity with ANS-IgG on 2D-gel identified at least five proteins with the following pI, Mr (isoelectric point, apparent molecular weight): (6.9-7, 49-59), (6.85-6.92, 37), (6.4, 54), (6.2-6.3, 53), (6.1, 53), (5.9, 51), (5.9-6, 46). They have yet to be characterized.

It would be interesting to have a cross-reactivity test by western blotting 2D gels of nasal septum D_3 and D_4 fractions with Rabbit Anti Rat Condyle IgG (AC-IgG). The undefined matrix proteins in D_3 and D_4 non-reactive with AC-IgG would be the potential unique proteins of the nasal

septum relative to the condyle. Nonetheless, to be complete, cross-reactivity with anti spheno-occipital synchondrosis IgG's and anti-epiphyseal plate IgG's needs to be performed to narrow down the spectrum of the potential unique proteins.

There is evidence of substantial cross-reactivity between the nasal septum and condyle cartilages as demonstrated by cross-reactivity tests between nasal septum cartilage extracts and anti-condyle raw antisera and vice versa. Thus the number of matrix proteins not reacting with either antisera appears to be small and would imply that the probability for isolating nasal septum or condyle unique proteins is slim. Furthermore, Hans (1981) in his experiments by injecting in growing rats the same anti-nasal septum antisera we are testing, showed statistically significant reductions in mid-facial growth, while the condyle was not altered. Both dot blots and Western blots demonstrated that the cross-reactivity between anti-nasal septum antisera and the condyle cartilage samples was substantial. This is consistant with Brigham's finding (1976) that only condylar cartilage demonstrated unique antigenicity. The nasal septum and synchondrosis were found to be immunologically identical. This suggests two possibilities. **1)** Anti rat nasal septum antisera cross reacts with the condyle and synchondrosis but is not cytotoxic. **2)** The anatomy of nasal septum allows anti rat nasal septum antisera to reach the reactive sites. Hans (1981) pointed out that anatomical vascularisation is more in favour of the nasal septum, with its rich vascular network, than the condyle, hence the nasal septum chondrocytes are more readily in contact with the injected antisera than those from the condyle. Therefore, repeating Hans' experiments, we would likely observe some

significant alteration of the midface upon injection of Anti Condyle antisera to growing rats.

We have found that link protein is mostly concentrated in the D_3 and D_4 fractions for the nasal septum, and in the D_3 fraction for the condyle. Link protein concentration in the nasal septum progressively increases from the D_1 to D_4 fractions.This observation was found by dot blots and confirmed by Western blot with monoclonal antibody 8A4. There is an abundance of link fragments in D_4, which is normal in high turnover tissue like the cartilage matrix of the growing rats used in this study. It was also reported that the occurence of these fragments seems to increase with age and may reflect natural degradation of the link proteins (Mort et al., 1985). Furthermore, it was suggested that the identification of these fragments may relate to the pathologic state of the proteoglycans within a given connective tissue. 2D gels under reducive condition and Western blotted with 8A4 clearly demonstrated the microheterogeneity of link protein by showing 9 to 10 adjacently defined bands. Link protein has been identified under the form of LP1, LP2 and LP3 with molecular weights of 51, 46, and 41 kD respectively, but different isoforms may exist. Under the conditions employed for isoelectric focusing, oligomerisation of link protein can occur, which explains the high number of adjacent bands (Baker et al., 1987). It has been found that human articular cartilage contains three link proteins, while rat chondrosarcoma only contains one link protein of approximately 43 kD. Other hyaline cartilages contain from one to three link proteins (Caterson et al., 1985). Monoclonal antibody 8A4 has been used in peptide map analyses and

has shown similar structural similarities in the link proteins obtained from rat, bovine, human and chicken hyaline cartilage (Caterson et al., 1985).

The role of link protein in stabilizing the interaction between the hyaluronate binding region of the proteoglycan and hyaluronate and which binds to both forming a ternary complex, could be explored by injecting mAb 8A4 in growing rats. The expected reactivity would be with the matrix protein fractions in D_3 and D_4 as the link epitopes in D_1 and D_2 require chondroitinase ABC digestion in order to be accessible.

Chondroitin-4-sulfate containing proteoglycan was revealed by its reactivity with monoclonal antibody 2B6 after chondroitinase ABC digestion and it was abundant in D_1 of the nasal septum. This was seen especially in the core protein, with much less in D_2 and none in D_3 and D_4 .

Monoclonal antibody 3B3 allowed us to map the chondroitin-6-sulfate which is higly abundant in D_1 of the nasal septum, minor in D_2 and devoid in D_3 . D_4 . Non-C_{ABC} treated samples showed a high molecular weight 3B3 epitope which favoured the existence of a subset of chondroitin-6-sulfate proteoglycans (i.e. reactive with mAb 3B3 without chondroitinase ABC digestion). Additional 3B3 epitopes might be revealed if D_4 were C_{ABC} treated. The 3B3 minus epitope is usually associated with growing tissue such as nasal septum cartilage and corresponds to an atypical terminal carbohydrate structure at the end of the chondroitin sulfate chain, i.e. a saturated hexuronic acid residue adjacent to 6-sulfated N-acetylgalactosamine (Caterson et al., 1985).

Monoclonal 5D4 was used to locate the keratan sulfate. We have found positive reactivity througout D_1 and D_2 and D_4. Cartilage proteoglycan

stains with 5D4, but this staining is very light in young rats which may be attributed to the low amount of keratan sulfate in young rat cartilage (Caterson et al., 1986). Superimposition of the 5D4 reactivity patterns on Coomassie stained gel would allow to discriminate the proteoglycans fractions from the matrix proteins.

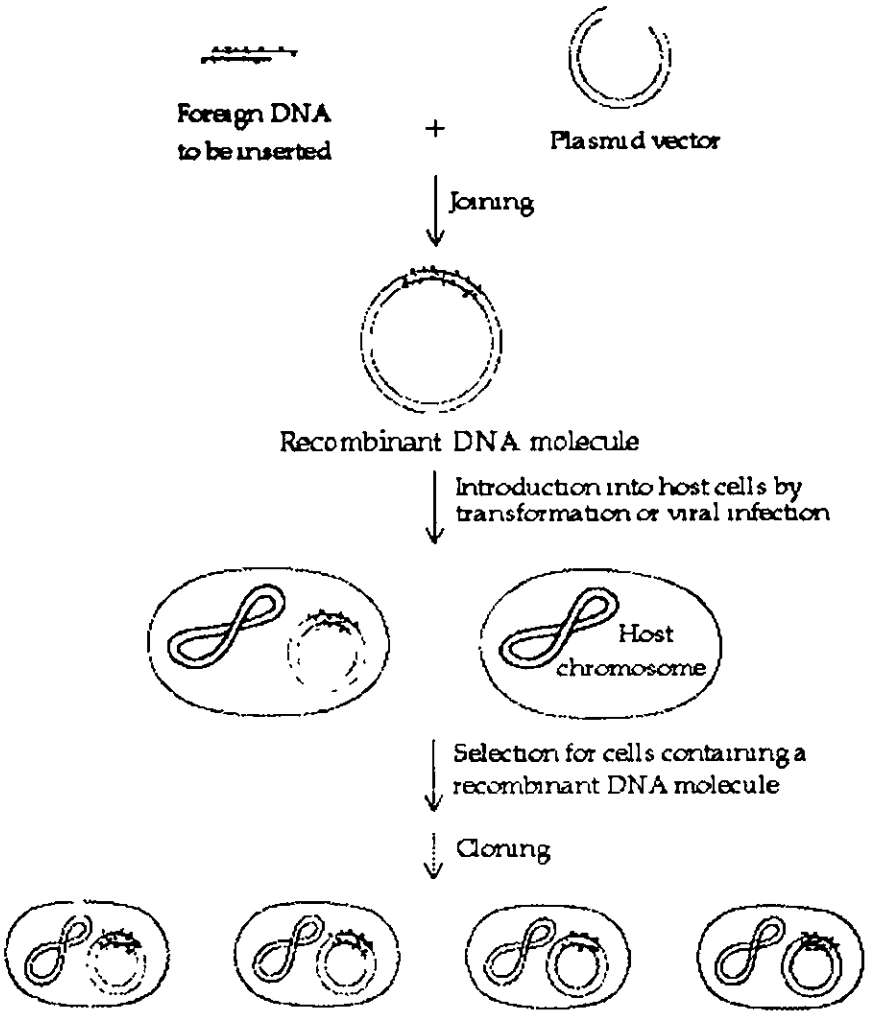
The results of this study show that the amount of matrix proteins is higher in the D_3 and D_4 than in the D_1 and D_2 fractions as demonstrated by dot blot with anti-PG (monoclonal antibody specific for proteoglycans). It appeared necessary to pretreat the samples with chondroitinase ABC to reveal the reactive epitopes on the proteoglycans, all of which is consistent with the findings of Caterson et al. (1985). The epitopes reactive with the antibodies might be sequestered by an interaction of the proteoglycan aggregates. Pretreatment may also be due to the design of the antibody itself which mirrors the conformation of the antigen for immunization. Caterson et al. (1986) reduced and alkylated the proteoglycan monomer to be used as immunogen for monoclonal antibodies specific to hyaluronic acid binding region. Subsequently, it appeared that none of the antibodies obtained reacted well with the whole antigens unless they were reduced and alkylated or enzyme pretreated to expose the epitopes. Another possible explanation for low reactivity with anti-PG is the dearth of keratan sulfate in young rat cartilage. Caterson et al. (1986) used monoclonal antibody chromatography: ^{35}S-labeled proteoglycans from 2 months and 12 months old rats were applied to an affinity column made using 5D4 (antikeratan sulfate monoclonal antibody). The pass through fractions were collected and ^{35}S-labeled proteoglycans bound to the affinity column by virtue of their

reactivity with 5D4 were eluted with 4M guanidine HCl. Quantitation of the radioactivity from the different fractions showed that proteoglycans of young rats contain very little keratan sulfate, only 5% vs 30% in much older rats.

Coomassie blue staining of 1D-gel showed that chondroitinase ABC digestion, besides exposing the epitopes by cleaving the chondroitin sulfates, is necessary for the large proteoglycans in D_1 and D_2 to enter the gel. Chondroitinase ABC depolymerizes both glucuronic acid and iduronic acid containing glycosaminoglycans at the β(1-4) bond. This depolymerization includes hyaluronate, chondroitin sulfate and the iduronate containing sequence of dermatan sulfate (Heinegard et al., 1987), producing disaccharides containing an unsaturated Δ^{4-5}-uronic acid at the non-reducing end. Care should be taken in the interpretation of the results concerning the molecular weight of the core protein shown by SDS-PAGE. Due to the high content of the remaining oligosaccharide stubs from the linkage regions of the glycosaminoglycans side chains, and N- and O-linked oligosaccharides, the core protein will migrate more slowly than expected, giving erronously high molecular weight. Thus, molecular weights obtained by SDS-PAGE can be used only for comparison. We have found in D_1 two adjacent heavy core proteins migrating significantly slower than the 200 kD marker protein. Sommarin et al. (1986) and Malemud et al. (1991) reported an apparent M_r of about 300 to 450 kD for these core proteins. Additionally, the benefit of screening by electrophoresis before and after enzyme digestion is the positive identification of the proteoglycan molecules which change mobility after digestion with specific enzymes (i.e. chondroitinase ABC). Any contaminating protein can also be revealed.

The major problems in 2D mapping reside in the extraction and handling of the vast amount of information. Thus a still widely used approach for protein identification is simply to superimpose two gels and match them as closely as possible manually. A more practical approach may be the procedure advocated by Jackson and Thompson (1984). They showed that gels that have been stained with Coomassie Blue R250 and destained in the usual way for the general detection of proteins can then be used for electroblotting to nitrocellulose membrane satisfactorily, provided that beforehand they are incubated at 4^{o}C for at least 1h in 0.025 M tris, 0.192 M glycine buffer containing 1 per cent SDS. In this case the Coomassie Blue staining zones in the gel were also transfered and bound to the nitrocellulose membrane allowing a staining of the blot before and after immunochemical detection.

Once the proteins localised, the production of monoclonal antibodies would allow more precise study of the role of a particular protein or group of matrix proteins in facial growth. Injection with monospecific antibodies in lieu of surgical excision would eliminate all the trauma associated with the "knife" and give a clean result directly associated with the experiment. Another alternative to using monoclonal antibodies would be to raise highly specific polyclonal antibodies which represent a viable solution considering the amount of work and technical expertise required for preparing monoclonal antibodies. Coomassie stained protein bands could be excised from the gel, then crushed and emulsified in complete Freund's adjuvant and injected in rabbit as for conventional antibodies preparation (Boulard and Lecroisey,



Synthesis and cloning of recombinant DNA molecules.
e.g. of host: E. Choli (Stryer, 1981)

1982). However, antibodies may also be formed against the acrylamide and Coomassie blue, and a better purification of the protein could be done by electrophoretic elution (Stearne et al., 1985; Kelly et al., 1983). During this procedure, the proteins undergo electrophoresis until the very end of the gel to be recuperated into a dialysis sac. The protein may be then recovered from the SDS and contaminating acrylamide polymers by precipitation with methanol following the procedure described by Stearne et al. (1985), and then emulsified in Freund's adjuvant for immunization or used for amino acid sequencing.

The determination of the peptide sequence is commonly done based on the Edman degradation chemistry. In this regard the sequencing is more efficient if the Western blot is performed on glass microfiber sheets which provide a better binding of the proteins eluted from the gel. Theses sheets are more resistant against the chemicals and solvents used in Edman chemistry (Vandekerckhove et al, 1986). Thus the specific unique antigens could be fabricated on a large scale for purpose of immunisation in rabbits. This is done by synthesizing the related genomes in the laboratory, and by covalently inserting the genes into the DNA of plasmids and viruses by using endonuclease and DNA ligase. Then the recombinant DNA molecules can be replicated and expressed in suitable host cells (e.g. E. Coli) (Stryer, 1981).

SUMMARY AND CONCLUSIONS

This study provides a biochemical method of analysis of rat cartilage matrix, and its interaction with an antisera, from the macroscopic to the molecular level.

The first goal of this research was to determine the matrix antigens reactive with anti nasal septal cartilage antisera (Anti-NS) known to alter rat midfacial growth. The Anti-NS antisera was reactive with large proteoglycans and link proteins, and small proteoglycans containing dermatan sulfate. 2D electrophoresis confirmed the results seen with 1D gels and in addition identified at least five reactive proteins in fraction D4 of the nasal septum, with the following pI (isoelectric point pH), Mr (apparent molecular weight in kilodaltons): (6.9-7, 49-59), (6.85-6.92, 37), (6.4, 54), (6.2-6.3, 53), (5.9, 51), (5.9-6, 46). These have yet to be characterized.

The second goal of this study is to identify the matrix proteins components of 11-day nasal septum cartilage in relation with cartilage characteristics found in the literature. The proteoglycans in the 11-day Sprague-Dawley rat are typical proteoglycans reported in the literature with these components:

- Heavy core protein of ≅ 300-450kD demonstrated by SDS-PAGE.
- Chondroitin-6-sulfate (mAb 3B3 positive).

- Chondroitin-4-sulfate / Dermatan sulfate (mAb 2B6 positive after Chondroitinase ABC digestion).

- Keratan sulfate (5D4 positive).

- Hyaluronate binding domain of ≅ 69kD demonstrated by SDS-PAGE.

- Link protein of ≅ 45-49kD demonstrated by SDS-PAGE.

The third goal of this study is to discriminate between nasal septum and condyle matrix antigens at the protein level. The protein patterns of nasal septum and condyle matrix proteins were grossly similar, but differences in quantity and quality between the two cartilages were apparent under 1D SDS-PAGE.

The matrix proteins binding with this antisera have been identified with their Pi and Mr. Future experiments should focus on the development of monospecific antibodies for each of the protein involved in order to characterize their individual interaction on rat facial growth

APPENDICES

I. Freezer milling procedure for cartilage samples.

II. Chequerboard for Dot Blotting.

III. Chondroitinase ABC digestion of cartilage samples.

IV. Preparation of 3-16% gradient gel for electrophoresis.

2270 Characterization of the Anti-adherence Agent κ-Casein.
A.M. VACCA-SMITH*, B.C. VANWUYCKHUYSE, L.A. TABAK, and W.H. BOWEN (Dept. of Dental Research, Univ. of Rochester, NY).

In this study the milk glycoprotein κ-casein, which we have shown to reduce the adherence of *Streptococcus mutans* GS-5 to saliva-coated hydroxyapatite (SHA), was characterized. κ-casein was fractionated by anion exchange chromatography and the four fractions obtained were tested in *in vitro* bacterial adherence assays. SHA was pretreated with either buffered-KCl, κ-casein, or κ-casein fractions. The treated SHA was incubated with radiolabeled streptococci, and bound bacteria were quantitated by liquid scintillation spectrometry. Of the fractionated κ-casein tested, fraction IV (FIV) reduced the adherence of *S. mutans* GS-5 to SHA by 71± 6% (SD) when present on the SHA surface. When FIV was added to SHA simultaneously with bacteria, adherence was reduced by 82±2%, as compared to controls in which bacteria were added to SHA in the presence of buffered-KCl. SDS-PAGE and silver stain analysis revealed one major protein band with a MW of 40,000, and two minor bands of 28,000 MW and 15,000 MW. Biochemical analysis of FIV revealed it to consist of 84±5% protein, 1.3±1% hexose, 6.4±2% sialic acid, 1.3% GalNAc, and 0.3% GlcNAc. Amino acid analysis revealed high amounts of Asx and Glx. Removal of sialic acid from FIV with and without subsequent trypsinization abolished the anti-adherence activity of FIV when present on SHA. These data suggest that a 40,000 MW glycoprotein fraction of κ-casein is inhibitory in the adherence of S. mutans GS-5 to SHA, and that sialic acid and protein are necessary for anti-adherence activity. Supported in part by USPHS grant DE07165 and DE07807; and by the National Dairy Promotion and Research Board and administered by NATIONAL DAIRY COUNCIL®.

2271 Cariogenicity Evaluation of Sweetening Agents Using an Modified ICT Device.
Y. IIJIMA*, N TAGUCHI and O. TAKAGI (Department of Preventive Dentistry, Nagasaki Univ. School of Dentistry, Nagasaki, Japan)

The aim of this *in situ* study was to determine the relative cariogenicity of sucrose and sorbitol tablets using the modified intra-oral cariogenicity test (ICT) of Koulourides. The first study was carried out with a 100% sorbitol tablet (A), the second tested a mixture of 95% sorbitol and 5% sucrose tablet (B), and the third tested a 100% sucrose tablet (C). In each study, 11 volunteers (age 19–25 years) wore non-removable appliances on which were mounted human enamel sections with a thickness of approximately 700 μm. Subjects wore the appliances for 3 days before taking various sweetening tablets to accumulate the plaque on the experimental surfaces and in the test phase took them 4 × per day for 7 days after meals and before sleeping. At the end of the 10 days experimental period, microradiographs of the enamel sections were taken and the degree of demineralization were assessed by next parameters; the mineral loss value (ΔZ) and the lesion depth (ld). Significant differences were observed between sorbitol tablets (A) and sugar-containing tablets (B, C). There were no lesions for sorbitol tablets. However, two types of lesions were observed; the only one case of surface softening lesion for B and two cases of subsurface lesions for C. Both parameters showed that 100% sucrose tablets were not always produce the highest level of demineralization. These results indicated that the lesion produced *in situ* were variable even in the same enamel sections and also in different subjects. This study was supported in part by Japanses Ministry of Edu. Sci Cul. Grant # 04304045

2272 Radiographic evaluation of skeletal growth in a new mutation (dense incisor, *din*) in the mouse. H.T. LOEVY*, A.F. GOLDBERG, S.C. MARKS JR., C. MACKAY, H.O. SWEET and M.T. DAVISSON (University of Illinois at Chicago; Veterans Affairs Medical Center, North Chicago IL; University of Massachusetts Medical Center, Worcester MA and The Jackson Laboratory, Bar Harbor ME).

We have examined skeletal growth in a new recessive mutation, dense incisors (*din*), in the mouse. Mutants (*din*/*din*) are distinguishable from normal litter mates (NLM) during the second postnatal week by their smaller size and grayer coat color. Radiographically mutants have dense incisors. Radiographs of the tibiae, femora and bisected heads of mutants and NLM were performed at 34,51 and 77 days using mammographic techniques, magnified, traced and compared. Superimposition of tracings showed that mutants were proportionately smaller that NLM in all dimensions. These data show that the *din* mutation in the mouse also affects general skeletal development to produce a proportionate dwarfism in addition to the delayed incisor eruption.

2273 Identification of Unique Extracellular Matrix Proteins in Rat Craniofacial Cartilages.
(F.T. NGUYEN, C.J. MALEMUD, AND M.G.HANS*, DEPARTMENT OF ORTHODONTICS, Case Western Reserve Univ., CLEVELAND, OHIO).

Previous studies (Hans 1981 Master's Thesis, CWRU, Hans et.al. IADR prog. abst 61:437,1982) demonstrated that it may be possible to control facial growth by targeting specific craniofacial cartilages. This study examined the protein structure of the extracellular matrix of rat nasal septum, condylar, and synchondrosal cartilage. Three to sixteen percent gradient SDS-PAGE electrophoresis and 2 dimensional gel electrophoresis were used to identify unique protein patterns among the various cartilage matrices. In addition, Dot blot analysis and Western Blot analysis were used to identify reactive matrix antigens. The protein patterns among the cartilages were grossly similar but some differences in quantity and quality between the nasal septal and condylar cartilages were identified. The gels for the synchondrosal cartilages were unlike the patterns observed for the other two cartilages. An antisera similar to the one used by Hans (1981) to modify rat midfacial growth in vivo was reactive with several large proteoglycans,link protein and several small proteoglycans containing dermatan sulfate. Dermatan sulfate content in the reactive proteins was confirmed because these proteins also reacted with monoclonal antibody 2B6 after digestion with chondroitinase ABC. Other specific monoclonal antibodies (1A4, 3B3, and 5D4) were also used to characterize the reactive bands. Two dimensional electrophoresis confirmed the results seen with the 1D gels for the nasal septal and condylar cartilage. In addition, at least five proteins with the following isoelectric points and molecular weights were identified. ([1] pI=6.9-7 MW= 49-59 kD ; [2] pI= 6.85-6.92 MW=37 kD ; [3a] pI=6.2-6.3 MW=53 kD ; [3b] pI=6.1 MW=53 kD ; [4] pI=5.9 MW=51 kD ; [5] pI=5.9-6.0 MW= 46 kD) These proteins have yet to be characterized.The results of these preliminary studies indicate that the possibility of developing monoclonal antibodies specific for each craniofacial cartilage exists. Further, these results indicate a potential to exploit these extracellular matrix interactions to determine which interactions are important for normal facial growth and development. Funded by a Grant from the AAO.

2274 Msx-2 Expression is Correlated with Non-Chondrogenic Regions of the Avian Mandible. H. HINA*, J. GLUHAK, & B. ROGERS (Univ. CT, School of Dental Medicine, Farmington, CT)

We have previously identified region-specific expression of Msx-1 and Msx-2 genes in the avian mandibular arch using in situ hybridization on sections with radioactive probes. In the present study we have reexamined the three dimensional localization of these mRNAs in chick craniofacial region (stage 14-30) using non-isotopic whole-mount in situ hybridization. The results of our whole mount in situ hybridizations are consistent with the localization of these mRNAs reported previously using radioactive probes and provide additional information regarding the three dimensional distribution of these mRNAs in the chick craniofasial region. The possible roles of these molecules in mandibular morphogenesis was investigated by examining the chondrogenic potential of the mesenchymal cells expressing the transcript of Msx-2 gene. Mandibular arches from stages 23 embryos were isolated. The middle region (mesenchyme expressing the Msx-2 transcript) was seperated from the lateral region. Cells from both regions were prepared for standard micromass culture. Mandibular mesenchymal cells isolated from lateral regions formed cartilage in micromass cultures whereas cells isolated from the middle region did not form cartilage. The results of these experiments indicate that region-specific expression of the Msx-2 genes correlates with medial non-chondrogenic regions of the mandibular arch.
Supported by NIH grant DE08682-08.

2275 Craniosynostosis and Mutation in the Human MSX2 Gene. M.L. SNEAD*, R.W JABS, X. LI, L. MA, W LUO, I. HAWORTH, I. KLISAK, R. SPARKES, M.L. WARMAN, J B MULLIKEN, U MULLER AND R. MAXSON (University of Southern California, Center for Craniofacial Molecular Biology, Los Angeles, CA, Johns Hopkins School of Medicine, Baltimore MD; Harvard University, Boston, MA; Justus-Liebig Universitat, Giessen Germany)

Normal development of the mammalian skull requires the coordinated growth of the component bones at the sites of articulation known as sutures. Craniosynostosis results when there is premature fusion of the calvarial sutures causing the skull to assume an abnormal shape. We have identified a homeobox containing gene termed MSX2 (Genomics 16 123-131 1993) that is expressed by cells of the developing skull, maxilla, mandibula, tooth, tongue, and suture, which we have mapped to human chromosome 5qter. An autosomal dominant form of craniosynostosis (Boston-type) has been described in a kindred which also maps to the distal long arm of chromosome 5, hence it is a good candidate gene for craniosynostosis. Using a polymorphic marker in the MSX2 intron we analyzed this kindred and demonstrate that MSX2 segregates with the disorder with no recombination (lod score 4 8) Sequence analysis of the mutated allele identified a histidine substituting for a highly conserved proline at position 7 of the homeodomain of the MSX2 gene exclusively in affected members. Molecular modeling reveals a major change in the geometry of the mutated homeodomain in a region implicated in protein to protein interactions. These results provide compelling evidence that this mutation in the MSX2 gene causes craniosynostosis and is the first report of a mutation in the homeobox gene associated with a human disease Supported by the National Institutes of Health and the National Institute for Dental Research.

2276 Immunohistologic Localization of Type IX Collagen during Intramembranous Bone Formation. K. TING*, and L NISHIMURA. (Harvard School of Dental Medicine, Boston, MA, USA)

The presence of an altered cartilage phenotype during *in vivo* intramembranous bone formation was previously reported using the tooth extraction socket created in rat alveolar bone (J Bone Mineral Res, In press). Cartilage collagen type II and IX mRNAs were expressed in this model. However, it was found that the α1(IX) mRNA was a short transcript from the down stream start site/promoter which is a different site in the α1(IX) gene from that used in hyaline cartilage. *In situ* hybridization showed that the α1(IX) collagen message was expressed by osteoblastic cells clustered near early bone matrix in the extraction socket. The purpose of this study was to investigate the presence and the fate of the truncated type IX collagen protein during intramembranous bone formation. Maxillary molars were extracted from 40-day-old male Sprague-Dawley rats and the alveolar bone including healing sockets were harvested at day 4 post-extraction. The specimens were fixed immediately in 10% buffered formalin, decalcified, and embedded in paraffin. Immunohistochemistry with monoclonal anti type IX collagen antibody D1-9 and B3-1 was carried out using a standard avidin-biotin complex/immunoperoxidase protocol. The results showed the presence of type IX collagen in three areas: 1) Over the early bone matrix where cells expressed type IX collagen message around the border; 2) Over the newly formed bone derived from the early bone matrix; 3) Near the border of well calcified bone where active remodeling seemed to be occurring. No type IX collagen epitope was found inside the matured bone matrix and, therefore, type IX collagen appeared to be present only during the bone formation period. Our results suggest that type IX collagen may play a possible role in organizing bone matrix during bone remodeling. (Supported by NIH grants DY08219 and AR36820)

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2262 A Promising Preventive Strategy for Infant Caries? Results of an Initial Study

P Weinstein*, P Domoto, M Koday, B Leroux. Univ of Washington School of Dentistry, Seattle, WA.

Following bilingual parental interviews at a clinic in Toppenish, Washington, 130 migrant farmworker children with a mean age of 17 months were examined with a light and mirror. Cotton swabs were used to collect plaque for a caries activity test (Cariostat) and a fluoride varnish (Duraflor) was painted on all the surfaces of four maxillary incisors. At six months 79 of the children received another examination and parents completed a brief questionnaire. Results using a one-sided McNemar's test for change in disease prevalence revealed a significant decrease in decayed and decalcified teeth ($p = .039$). Disease prevalence decreased from 41 to 29 percent. The pattern of decrease was the same for all four teeth. Eighty-six percent ($^{130}/_{151}$) of all sound teeth remained disease free and 56 percent of the decalcified teeth ($^{55}/_{98}$) remineralized. Parental report of bottle use as the child falls asleep was associated with development of disease at 6 months among children with no disease at baseline (Fisher's exact test, $p = .014$). The Cariostat was associated with subsequent disease after adjusting for dental age using the Cochran-Mantel-Haenszel test ($p = .002$). Controlled trials with young children are underway. Results suggest that while fluoride varnish reduced disease in young children, future work must attend to behavioral risk factors.

This study was supported by Grant P30 DE09743

2263 Methodological Refinements in the Assessment of Risk of Caries Onset.

R.J BILLINGS and H.M PROSKIN* (Eastman Dental Center, Rochester, New York, USA)

As part of an ongoing longitudinal study, clinical, microbiological, and salivary chemistry parameters were measured on 240 children in an optimally fluoridated (F) community and an 178 children in a fluoride-deficient (FD) community at each of four semi-annual examinations. Children were six years old and caries-free at the initial examination. The goal of this investigation has been to model the occurrence and non-occurrence of caries onset at a particular examination on the basis of parameters measured previously. A key feature of this process is the determination of the time point at which caries is said to occur. Of particular concern has been the extent to which subjects who do not manifest any signs of clinical caries may actually be in the early stages of the caries process. Such a circumstance could serve to impact on the microbiological and salivary chemical parameters measured in this study. Thus, as a refinement in the modeling methodology, a reassessment of the caries status of study subjects was made, with subjects only being classified as caries-free at a given examination if no caries or fillings were presented at the subsequent examination, as well. As has been done previously, subjects were classified as "caries" if any active caries was observed at a study exam. Stepwise discriminant analyses were employed to suggest models for F and FD which might be employed to differentiate between the subjects classified as "caries" or "no caries" according to this refined method. The sensitivity values of the resultant models were 92% and 71% for F and FD, respectively; for both communities, the specificity was roughly 80%. Supported by NIH/NIDR Grant DE08948.

2264 Structure and pH of 3 and 5 Day Old Dental Plaques Developed on Glass and Enamel *in situ*.

O. FEJERSKOV*, V. BAELUM and A. KÜSHLER (Royal Dental College, University of Aarhus, Denmark).

The aim of the present study was to test the hypothesis that the structure and pH-response of dental plaque formed on either enamel or glass would be different. Acrylic flanges containing glass and enamel specimens were mounted on buccal surfaces in the lower jaw of 9 volunteers for 3 and 5 days during which plaque was allowed to accumulate. The pH response to a controlled sucrose rinse was measured for 60 min. following the rinse using microtouch electrodes. Subsequently, all specimens were fixed and embedded in Epon for light and electron microscopy (a total of 36 glass and 36 enamel specimens). There were no statistically significant differences between the mean pH responses of plaque accumulated on glass and enamel after 3 and 5 days. However, minimum pH ($\bar{x} = 4.94$-5.15) was reached more rapidly and the subsequent return towards resting values occurred earlier in 3-day old plaque. The thickness and structural composition of the microbial deposits varied extensively between individuals - but within individuals no significant differences were observed between plaque grown on glass or enamel. It is concluded that glass substrates are as useful as enamel surfaces for studying plaque metabolism *in situ*.

2265 Mutans Streptococci in Margins of Restorations after Intermittent Chlorhexidine Treatment.

C. WALLMAN*, B. KRASSE and D. BIRKHED (Department of Cariology, University of Göteborg, Sweden).

Treatment with chlorhexidine (CHX) gel results in a considerable reduction of mutans streptococci in margins of restorations. However, the effect is limited in time. In an attempt to prolong the suppression of mutans streptococci, intermittent CHX gel treatment, based on microbial examination, was tested. Eighteen subjects, nine in a control and nine in a test group, were treated with 1% CHX gel in individually designed applicators 3x5 min on two consecutive days. Saliva and plaque samples from margins of restorations were cultivated on Strip Mutans (Orion Diagnostica) at baseline and up to 20 weeks after the CHX treatment. When mutans streptococci were detected in the samples, the gel treatment was repeated on the patients in the test group up to 12 weeks after the start of the study. In the nine subjects in the control group who only received the initial treatment, mutans streptococci were back to pretreatment levels in margins of restorations and in saliva after 8 weeks. In the test group, the proportions of mutans streptococci were significantly reduced in margins of restorations ($p<0.05$) at the 16-week sampling occasion compared to baseline. There was also a reduction of the salivary counts of these microorganisms ($p<0.01$). Thus, intermittent treatment with CHX gel based on microbial examination prolonged the reduction of mutans streptococci in margins of restorations compared to conventional treatment.

2266 Influence on Dental Caries of Enamel Hypoplasia and Colonization of Mutans Streptococci in Rural Chinese Children 3 to 5 Years Old

Y LI* and J.M. NAVIA (Schs. of Dentistry and Public Health, U. of Alabama, Birmingham, AL.)

The purpose of the present study was to determine the epidemiology of enamel hypoplasia (EHP) and dental caries in Chinese children and to assess the determinants of the high prevalence of caries in their primary dentition. A total of 1,344 3- to 5-year-old children were examined clinically for caries; mutans streptococci (MS) in saliva was assayed using Bratthall's Strip test. A modified DDE index was developed to evaluate hypoplastic defects in primary teeth. There are three major findings: (1) The prevalence of EHP was 22.3% among the population studied. The occurrence of this defect was associated significantly with males (OR=1.5; 95% CI=1.2-1.8), low birth weight (OR=2.0; 95% CI=1 1-3.8), and prematurity (OR=3 8; 95% CI=1 5-9.8); (2) MS was detected in 94.7% of 486 children sampled. The difference in concentration of MS from the children's saliva was significantly associated with presence of EHP ($p<0$ 001), (3) A high proportion of children with dental caries was observed in the population studied (82.3%). Children who had EHP had 2-5 times more dental caries ($p<0$ 001). Results suggest that the time between mid-gestation and the first 6 months of life is the most critical period for developing EHP; morphologic alterations and irregularities of enamel surfaces may promote a greater colonization of MS in the oral cavity; EHP could be an important predictor for caries risk assessment in the general population. This study was supported by the UAB John Sparkman Center for International Public Health Education.

2267 Effect of Plaque Fluid Fluoride on Lactic Acid Production After Sucrose.

Y. MAO*, G.L. VOGEL, C.M. CAREY (ADAHF-FRC, Gaithersburg, MD).

The purpose of this experiment was to examine the relationship between F, pH and lactate in plaque fluid (PF) before and after a sucrose challenge and a F rinse. Subjects (6 to 8) abstained from brushing for 48 h and fasted overnight before the study. A 228 ppm F 2-component rinse (JDR, 1992, p448) was administered as a 1 min rinse at 1 h or overnight before a 1 min 10% sucrose challenge. Molar plaque samples (≈ 150 µg required per sample for analysis) were collected before and 5 min after sucrose administration. Control experiments without the F rinse were also performed. PF (≈ 0.05 µL) separated from these samples was analyzed for F and pH with microelectrodes (JDR, 1990, p1316) while lactate was measured by capillary electrophoresis. Results were (F µmol/L, lactate mmol/L, n = 19 to 31):

	Control		12 hrs after F Rinse		1 hr after F Rinse	
time	0	5	0	5	0	5
F	8.8±6.3	7.2±5.1	19±12	17±15	65±42	135±85
pH	7.03±0.30	5.51±0.39	7 04±0.27	5.59±0.44	6.50±0.35	5.73± 024
Lactate	1.9±1.8	23.6±9.3	2.6±2.4	22±10.	1.5±1.5	16±12

Results indicate: (1) The two-component rinse induces a large persistant increase in PF F ($p<0.001$). (2) The sucrose challenge given 1 h (but not 12 h) after the F rinse produced a large release of F ($p<0.001$). (3) Relative to the control, a small decrease in lactic acid production and a small increase in 5-min pH were associated with the 1 hour post sucrose F release ($p<0.05$). Supported by NIDR grant DE04385, ADAHF, and NIST.

2268 Evaluation of plaque pH changes following oral rinse with eight infant formulas.

C. Sheikh* and P. Erickson (Pediatric Dentistry and the Clinical Dental Research Center, University Of Minnesota, Minneapolis, Minnesota, USA).

Baby Bottle Tooth Decay (BBTD) continues to be a major cause of debilitating and costly dental pathology, affecting 6% of children under 3 years old. Inappropriate feeding habits have been identified as the major factor associated with the development of BBTD. This study sought to compare plaque pH changes following oral rinse with eight different infant formulas. These formulas were representative of the most commonly used infant formulas from two companies; Mead Johnson And Ross Pharmaceuticals. These eight formulas were also representative of four categories. (1) formulas with iron, (2) formulas with low iron, (3) soy formulas, and (4) protein hydrolyzate formulas. Ten percent sucrose was used as the control. The sample size consisted of nine subjects for nine trials. Adult subjects were to asked to abstain from oral hygiene for 24 hours and to fast for a minimum of two (2) hours prior to rinsing with the formula. Plaque was sampled from maxillary buccal surfaces before and after a 5 cc rinse of formula for one minute. Plaque was then dispersed in 50 microliter deionized water and pH changes were monitored for one hour. A significant difference was demonstrated in the pH changes following oral rinse with protein hydrolzate or soy formulas when compared to the other two categories. On average, after rinsing with protein hydrolyzate formula, plaque pH reached a minimum of 5.24, with an average pH drop of 1.29. After rinsing with soy formulas, plaque pH reached a minimum of 5.21 with an average drop of 1.33. We conclude that infant formulas differ significantly in their ability to alter plaque pH. Additional trials may be necessary to further identify the significance of these pH changes. However, these results suggest that the development of less acidogenic formulas may be beneficial to reduce the incidence of BBTD.

2269 Anticaries mode of action of casein derived phosphopeptides

A J ROBERTS*, C A TAYLOR, D T LITTLEWOOD and C P REAY (Unilever Dental Research, Bebington, UK).

Phosphopeptides derived from the digestion of sodium caseinate by trypsin have been reported to display anticariogenic activity. The aim of this study was to investigate the mode of action of this agent, and to investigate the potential activity in situ when delivered via a dentifrice. Pellicle covered, partially demineralised bovine enamel samples were treated with a solution of a calcium phosphate complex of a mixture of casein derived phosphopeptides. The samples were subjected to a remineralising solution, spiked with ^{45}Ca, for 16 hours, and then analyzed for ^{45}Ca uptake. Treatment with the phosphopeptide solution was found to reduce the uptake of calcium by the demineralised enamel by 80.1%. Conversely, the retention of ^{45}Ca initially introduced into the enamel, upon exposure to demineralising conditions was found to be 60% greater following treatment with the phosphopeptide. A dentifrice containing the casein derived phosphopeptide mixture was compared to a placebo dentifrice using an intra oral model. 30 adult subjects participated in this crossover study in which sound bovine enamel blocks were attached to partial dentures and exposed in the mouth for three weeks. During this period, subjects brushed with their assigned test dentifrice, and also immersed the enamel blocks in 5% aqueous sucrose solution for 5 minutes 3 x daily. At the end of the test period, the blocks were removed and the mean surface hardness was determined. Both treatments resulted in significant ($p<0.001$) surface softening of the enamel. However, treatment with phosphopeptide reduced the softening by 26.5% (s.d = 49.97) Knoop Hardness units, a reduction of 20.7%. This difference between treatments was statistically significant ($p< 0.01$). It is concluded that the anticaries activity of casein phosphopeptides is due to the inhibition of enamel demineralisation, and that this effect is manifested in vivo in a reduced enamel surface softening under cariogenic challenge.

APPENDIX I

Freezer Milling Procedure for Cartilage Samples.

1- Put the cartilage samples in Gey's Balanced Salt solution (Gibco Lab, Life Technologies Inc., Grand Island, NY 14072).
2- Dice the cartilage into 1-2mm^2 pieces.
3- Be sure to draw off all the Gey's Balanced Salt solution.
Divide the cartilage into 2-3 portions (depending upon the initial amount).
Put on ice.
4- Fill up freezer mill with liquid nitrogen to line indicated (Spex Freezer/Mill, Spex Industries Inc., Metuchen, NJ).
Also fill up cryothermos as well.
5- Put end plug in plastic sample tube manually.
Load cartilage on the side of the tube, using spatula.
Put impactor in center of tube. Avoid to touch cartilage, if not it would stick together with the impactor, and would give an incomplete milling).
Put the top plug (with nipple) on top of tube manually.
6- Place tube into freezer mill filled with liquid nitrogen to fill line (pay attention to the protruding end which should be the one with the nipple plug, for prehension with a special tool, once done).
7- Allow the tube to sit in the liquid nitrogen for 2-3 minutes.
8- Set freezer mill timer to 1.5 minutes. Start and slowly increase the impaction speed from minimum to maximum by the 1 minute remaining time.
9- Remove tube using special tool, and place it in the black cap removal device.
Tighten end of tube down and then remove cap by twisting it off using removal tool.
10- Remove impactor using forcep and place on a clean paper towel.
11- Add 5ml of 4M guanidine HCl in 0.1M sodium acetate buffer, pH 5.8 containing protease inhibitors (100 mM 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 10 mM Na_2 EDTA, 20µg/ml ovomucoid) . Use a disposable pipet.
Try to get powdered cartilage dissolved into solution.
Fill Guanidine HCl to near top of the tube.
Pour into Ehrlenmeyer flask setting on ice.
Pour more Guanidine HCl into tube if needed to wash out all the cartilage (Guanidine HCL volume is not crucial because the final extract will be lyophilized).

Finally put the impactor back into the tube, and pour in Guanidine HCL to wash the cartilage powder from the impactor

12- Repeat steps 5-11 with other aliquots of cartilage.

Matrix Protein Extraction.

13- Extract overnight at 4°C on magnetic stirrer.

14- Centrifuge the extract at 2,400 rpm for 10 minutes (SS-34 rotor, Sorvall S6-B centrifuge) to remove the debris.

15- Dialyze the extract vs distilled demineralized H_2O for 24hrs at 4°C on magnetic stirrer, using 6-8K cut off membranes (Medical Spectrum Industries, LA, CA). Water was changed twice.

16- Centrifuge the dialysates at 15,000rpm, for 10 minute (SS-34 rotor, Sorvall S6-B centrifuge) to generate the 15-K supernate and 15-K pellet fractions.

17- Freeze dry the 15-K supernate fractions.

Keep the 15-K pellet fractions at -70°C for later comparison.

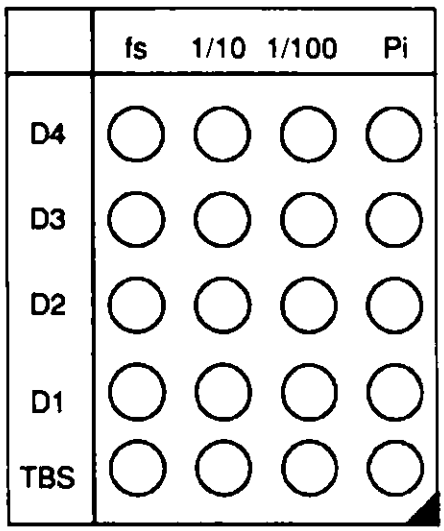
18- Proceed with CsCl centrifugation.

19- Dialysis against distilled demineralized H_2O for 24h, with 6-8K cut off membrane tubing.

20- Proceed with uronic acid and protein analysis.

APPENDIX II

Chequerboard for native Dot Blotting.



Control with no antigen occupies the last column

Control with no antibody occupies the last row

fs: full strength of sample
TBS: Twist Buffer Saline

Pi: Pre-immune sera

Full strength of sample is adjusted at 10µg of protein / 100ml for D4 to D2.
Full strength D1 contains 10µg of uronic acid / 100ml, as D1 is mostly rich in proteoglycans.

1- Pre-wet the nitrocellulose membrane in TBS
2- Load samples as designed.
3- Turn vacuum on. Suck samples through the nitrocellulose membrane.
4- Rinse each well with 100µl TBS, then turn the vacuum on to suck through.
5- In Petri dishes, incubate the nitrocellulose membrane with 3% BSA (bovine serum albumin) in TBS-Tween, in order to block the remaining sites on the nitrocellulose paper. 30 min on shaker at room temperature.
6- First antibody: in separate Petri dishes, incubate the nitrocellulose membrane, with 4ml of the antibody to be tested, on shaker at room temperature for 1 hour.
7- Wash three times 5 minutes in TBS-Tween.
8- Second antibody: in Petri dish, incubate on shaker at room temperature for 1 hour, with :

- Alkaline Phosphatase Goat Antimouse IgG, diluted to 1/750 with TBS-Tween: 2µl Goat Antimouse IgG + 15ml TBS-Tween.

9- Wash three times 5 minutes in TBS-Tween.
10- Rinse twice with Alkaline Phosphate Buffer.
11- Color development: in Petri dish, incubate up to 30min in dark room with:
• Alkaline Phosphate Substrate: 12ml Alkaline Phosphate Buffer + 80 µl NBT(Nitro Blue Tetrazolium)+ 40 µl BCIP(5-Bromo-4-Chloro-3-Indolyl Phosphate).
12- Wash with distilled H_2O and air dry.

- Tris Buffered Saline (TBS): 20mM Tris
500mM NaCl
pH 7.5

- TBS-Tween: 20mM Tris
500mM NaCl
0.05% Tween-20
pH 7.5

APPENDIX III

Chondroitinase ABC Digestion of Cartilage Samples.

1- Samples were diluted as required by the design of the experiment in 1ml vials (e.g. 20µg of protein sample).

2- Samples were freeze-dried overnight.

3- Add buffer: 50 µl for each vial (25µl / 10µg of protein)

4- Add an equal volume of chondroitinase ABC (2.5U/ml, Products for Life Science), made up in the same buffer, was added to each sample.

5- The samples were incubated at 37°C for 3 hours.

6- The reaction was stopped by boiling for 5 min.

7- Any precipitation was removed by centrifugation.

8- Full strength volume before lyophilisation was reconstitued by adding the [initial volume before lyophilisation - 100µl] of TBS (if it were for Dot Blotting) or Gel Sample Buffer (if it were for Isoelectric Focusing gel).

Buffer:

{ 0.1M sodium acetate + 0 1M Tris } adjust pH to 7.0 with acetic acid

10µg/ml pepstatin
5mM phenymethylsulfonyl fluoride
10mM N-ethylmaleimide
10mM disodium ethylenediamine tetraacetate
100mM 6-aminohexanoic acid
5mM benzamidine hydrochloride
20µg/ml ovomucoid

APPENDIX IV

Preparation of 3-16% Gradient Gel for Electrophoresis.

- For 4 slab gels:

	3%	16%
Tris(1.5M Tris base) (pH 8.8)	20ml	20ml
Acrylamide/Bis (30%T, 2.7%C_{bis})	7.5ml	40ml
Sucrose	-----	10.20 g
	Filtering and Degassing	Filtering and Degassing
10% SDS	0.8ml	0.8ml
1%Ammonium Persulfate	4.0ml	2.0ml
H_2O	to 80ml	to 80ml
	Remove 35ml (for 2 slabs) of each solution	
TEMED	18µl	9µl
	Fill the corresponding resevoirs of the gradient maker Start magnetic stirrer and pump	

- **Acrylamide/Bis, 30%T, 2.7%C_{bis}, to store at 4°C in the dark:**

Acrylamide	58.4g
Bis	1.6g
H_2O	to 200ml

Acrylamide is toxic, therefore make sure to wear gloves.

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