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ANTIGENIC DETERMINANTS IN CARTILAGE OF THE SPRAGUE DAWLEY RAT

by

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Abstract

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Whole cartilage and cultured chondrocytes obtained from the mandibular condyle, nasal septum, and epiphyseal plates of 9-11 day old Sprague Dawley rats were examined by SDS gel electrophoresis and silver stain for the presence of unique proteins. Cell membrane proteins from the chondrocytes of the mandibular condyle, nasal septum, and epiphyseal cartilage were found to be similar.

The extra-cellular matrix of the three cartilages examined as found to contain several unique protein bands. For the cultured extracellular matrix of the mandibular condyle unique bands were identified in the 38,000 and 43,000 dalton range. For the nasal septum cultured extra-cellular matrix, unique bands were observed in the 40,000 and 50,000 dalton ranges. And for the epiphyseal cultured matrix unique bands were observed in the 32,000 and 78,000 dalton ranges. Protein differences were

also found between freshly isolated cartilage matrix and cultured chondrocyte matrix.

Because unique protein bands were present in the mandibular condylar matrix, the nasal septal matrix and the epiphyseal cartilage matrix it should be possible to produce monoclonal antibodies to each of these cartilages. Anti cartilage antibodies would be an important analytic tool for studying the role of each of the craniofacial cartilages in facial growth.

DEDICATIONS

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INTRODUCTION

The prospects for clinical control of craniofacial growth have been a subject of intense interest in the field of Orthodontics for many decades. Early practitioners manipulated dentoalveolar structures in hopes of producing ideal facial features. Because efforts to influence facial development solely through the dentoalveolus were not successful, a genetic paradigm developed and the concept of a predetermined pattern of growth gained acceptance.

Gradually, research shifted to epigenetic factors that influence the growth process. The functional matrix and the concept of growth modification through functional alteration of the oral environment have been tried with limited success. Although various factors have been demonstrated to have an influence on facial growth, the precise signals which initiate cell division and differentiation have, as yet, remained undiscovered. Although the concept of cartilaginous "growth centers" responsible for controlling facial development has been discarded, experimental evidence still supports the concept that the nasal septum and mandibular condylar cartilages play significant roles in the normal development of the face.

Sophisticated biochemical and immunohistochemical techniques now allow the analysis of the molecular structure of cartilage. Brigham (1976) first reported that antigenic differences exist among craniofacial cartilages. The presence of antigens unique to each craniofacial cartilage led later investigators (Palmer, 1978; Hans, 1981) to explore the possibility of

producing anticartilage antibodies that could be used to control facial growth. In 1981, Hans demonstrated that cytotoxic anticartilage antibodies could be produced in rabbits against the nasal septum of rats. Injection of these anti-nasal septum antibodies caused significant alterations in midfacial growth. The antibodies produced in these first experiments were directed toward extracts of whole nasal septum, mandibular condyle and epiphyseal cartilage. To specifically evaluate the respective roles of each craniofacial cartilage in facial growth requires a more specific antibody than is presently available. However before antibodies specific to each cartilage can be developed, one or more unique antigens (proteins) must be isolated from each cartilage.

Monospecific anticartilage antibodies will provide a method by which cartilage growth can be altered without disturbing adjacent tissues. The use of antibodies to selectively kill cells eliminates the need for surgical manipulation which by necessity, damages surrounding muscle, bone, vascular and neural tissues.

Antigenic determinants are proteins and other molecules that are capable of eliciting an immune response. These unique proteins may be present on the cell membrane or in the extracellular matrix of cartilage. Potential antigenic determinants in the cartilage extracellular matrix are proteoglycan core protein, link protein and various major and minor collagen sequences. In addition, glycosaminoglycans such as keratan and chondroitin sulphate and other minor non-collagenous proteins have been implicated as cartilage antigens (Poole et al., 1980; Champion et al., 1983). Chondrocyte membranes also contain potential antigenic sites in the form of various membrane bound proteins.

The purpose of the present study was to examine the nasal septal, mandibular condylar, and epiphyseal cartilages for the presence of unique proteins. These unique antigens may then be utilized for the production of monospecific anticartilage antibodies. Condylar, nasal septal, and epiphyseal chondrocyte membrane proteins obtained from cells grown in monolayer cultures, and from whole tissue were compared. Chondrocyte membrane proteins were also examined. Membrane proteins were obtained from freshly isolated cells which had been allowed to recover from the isolation process for both twelve and twenty four hours, as well as from cells grown in culture. Chondrocytes grown in culture were also examined for morphologic differences.

The identification of unique proteins in each cartilage is the first step in the production of monospecific anticartilage antibodies. The ultimate goal of this line of research is to allow dose dependent, tissue specific, control of the facial growth process. Before such specific growth control can be realized, specific targets (antigens) must be identified. Once the targets have been identified, monospecific antibodies can be created to selectively attach to their target antigens and produce cytotoxic effects. Depending on the amount of antibody used and the site of attack analysis of the differential input of each craniofacial cartilage to facial growth can be determined. In essence, growth modification and control can be effected at the cellular level allowing a precision that is unlikely to be achieved with mechanical devices or surgical intervention.

LITERATURE REVIEW

The role of cartilage in the growth of the craniofacial complex has long been the subject of much controversy. Experimental manipulation of the craniofacial cartilages has included transplantation and extirpation. These methods have yielded wide variations in reported results. More recently, molecular studies of cartilage have lead to great advances in knowledge regarding the structure, function and organization of the cellular and extracellular matrix components of cartilage. As in transplantation and extirpation studies, molecular studies too, exhibit wide variation in reported results. As methodology for molecular studies becomes more sophisticated, differences become apparent between cartilages studied under different conditions. One must consider whether the cartilage was studied in vitro or in vivo, it's anatomic location, the species, and the age of the subject. Cartilage can no longer be considered a homogeneous tissue. Cartilages are different with respect to histologic organization, composition of matrix components, phenotypic expression of cellular components and cellular morphology.

HISTOLOGY OF CARTILAGE

Cartilage is a specialized form of connective tissue composed of cells, fibrous macromolecules and ground substance. Its distinctive properties include low metabolic rate, avascularity, capacity for continual interstitial and appositional growth, high tensile strength coupled with

resilience and elasticity (Webster S.S. Jee 1983). Classification of cartilage in a functional sense, is three fold: (1) protection and support of related non-skeletal tissue and organs, (2) articulations between skeletal elements, and (3) certain dynamic processes related to skeletal growth (Moss and Salentiju, 1983). Microscopically cartilage is classified as hyaline, elastic, fibrous, or calcified. As more information becomes available classification becomes increasingly elaborate and dependent on numerous factors such as species, age and anatomic location. In general cartilage is composed of cells (chondrocytes) which secrete an elaborate matrix consisting of collagen and ground substance. Chemical analysis of the ground substance reveals three types of glycosaminoglycans: hyaluronic acid, chondroitin sulphate, and keratan sulphate. Chondroitin sulphate and keratin sulphate subunits are attached to a core protein to form Proteoglycan monomeres (fig. 1). Proteoglycan monomeres in turn are attached to hyaluronic acid chains with the aid of link protein to form the basic components of Hyaline cartilaginous matrix (fig. 2). There are numerous major and minor collagen sequences present as well as many non-collagenous matrix proteins. The relative composition of each varies from one anatomic location to the next. By weight, cartilage is mostly water. In fact, from 60 to 78% of the cartilage matrix is fluid and contributes to the role of cartilage as a supporting connective tissue.

Cartilages examined in this study were derived from the mandibular condyle, epiphyseal growth plates and the nasal septum of young rats. Each of these cartilages exhibits distinct histologic features and requires separate discussion.

FIGURE 1

Basic Hyaluronic Acid & Proteoglycan Monomeres

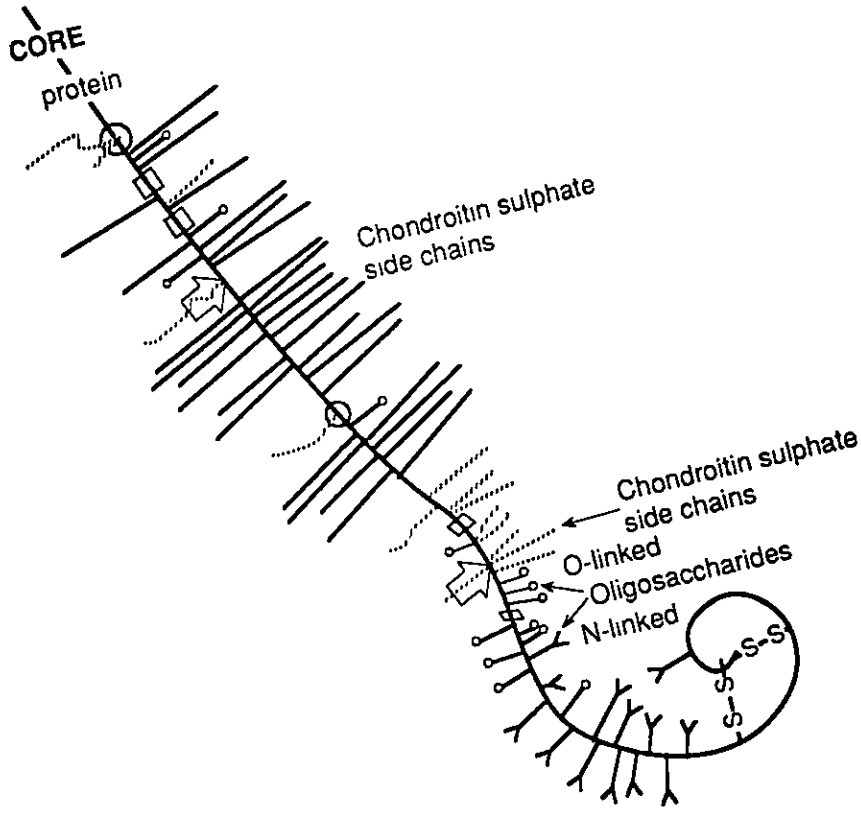
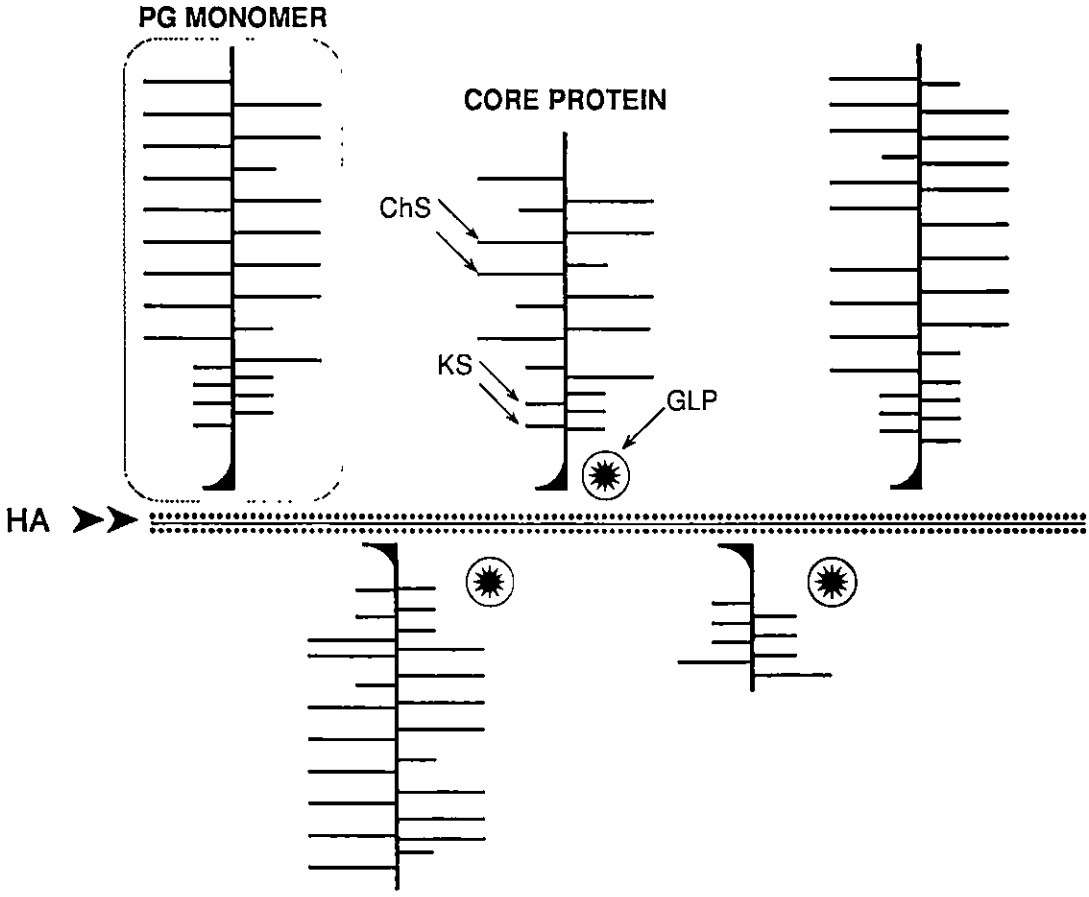


FIGURE 2
Proteoglycan Monomere



MANDIBULAR CONDYLE

Histologically, the cartilage covering the mandibular condyle is classified as fibrocartilage. Embryologically, the entire cartilage mass contained within the condyle is classified as a secondary cartilage. The condylar cartilage originates from the ectomesenchyme of the neural crest (Hoden 1975) and fuses to the body of the mandible at about twelve weeks in utero (Baume 1962). It is capped by a highly condensed fibrous layer. The cells composing the mandibular condyle are organized into four zones: (1) A resting zone composed of flat undifferentiated cartilage cells, covering the articular surface; (2) A transitional zone composed of proliferating cartilage cells; (3) A hypertropic zone composed of cells secreting extracellular-matrix; and (4) An erosive zone, marking the onset of calcification. The zones of the mandibular condyle are not arranged in linear columns, as typically described for epiphyseal cartilage of long bones. Instead they are rather haphazard and the transition from one zone to the next is generally regional instead of distinct. This irregular arrangement of the mandibular condylar cartilage has lead investigators to term this cartilage "embryonic cartilage"or secondary cartilage (Durkin 1979).

NASAL SEPTUM

The cartilage of the nasal septum originates from the mesoectoderm of the frontal process. It forms the most anterior part of the chondrocranium in the fetus. The nasal septal cartilage is considered to be a primary cartilage because it develops early as part of the embryonic cartilaginous endoskeleton. It is a midline structure in the nasal cavity and is bounded

posteriorly and superiorly by the ethmoid bone and posteriorly inferior by the vomer. Anteriorly the septum is attached to the premaxilla and the nasal bones (Young 1960). Histologically the cartilage of the septum reveals irregular distribution of chondrocytes. The central portion of the septum contains sparse numbers of chondrocytes arranged in linear columns in contrast to the greater number of chondrocytes in haphazard arrangement on the periphery of the developing septum. It is the precursor to bones that will develop in the suprapremaxillary area (Vetter et al., 1984). Distinct zones are not visible in the nasal septum as opposed to the mandibular condyle and epiphyseal cartilages. Large amounts of matrix separate the cells of the nasal septum which contains a large number of flattened cells (Scott, 1953). Due to its central position, the role of the nasal septum has been the subject of much debate. (Scott, 1953) argued that the growth of the septum was the driving force in the growth of the midface, while Moss (1968) contended that the septum plays only a passive role in facial growth with the soft tissue being the primary controlling influence. These debates have led others to manipulate the septal cartilage in an attempt to determine its precise role. Sarnat (1966) removed varying amounts of septal cartilage in growing rabbits and found the postoperative effect on midfacial growth to be proportional to the size of the defect created by surgical manipulation. However, Latham and Burston (1966) reported that in human subjects devoid of some or all of the nasal septum at birth, facial growth proceeded normally. From these studies, two schools of thought developed. One theory contends that the nasal septum functions as a midline supporting structure and plays only a passive role in facial growth. A second competing theory contends that

the nasal septum is the driving force in midfacial growth, and determines the extent and direction of the growth of the midface.

EPIPHYSEAL CARTILAGE

The cartilage of the epiphyseal growth plates of long bones has been studied extensively and most information available on cartilage histology is based on these studies. The cartilage of the epiphyseal plate is hyaline cartilage. Hyaline cartilage forms the initial skeleton of the fetus. It is the precursor to bones that will develop by the process of endochondral ossification. Hyaline cartilage forms a template that is transformed into bone during the growth process. At the cessation of growth, epiphyseal cartilage is present only at the articular surface of long bones and functions as the articular surface. The hyaline cartilage of the epiphyseal plate develops from mesenchymal tissue. Early mesenchymal cells proliferate to become chondroblasts. Chondroblasts then begin to secrete large amounts of matrix material surrounding and entrapping themselves in lacunae. Once trapped in lacunae, the cells are termed chondrocytes. Chondrocytes continue to secrete matrix into the extracellular space and become increasingly separated. This separation process is termed interstitial growth. The mesenchyme surrounding the developing cartilage condenses into a special layer toward the perichondrium. The inner layer of the perichondrium contains cells which during embryonic life continually give rise to new chondrocytes which also produce extracellular ground substance. It is thought that the ability of perichondrium to give rise to viable chondrocytes remains into adult life

(Bloom and Fawcett, 1970). Histologically hyaline cartilage characteristically appears as chondrocytes arranged in linear columns separated by extracellular matrix. There is an orderly progression in the direction of growth of the bone. The outer most zone consists of proliferated chondrocytes flattened and closely arranged through the length of the columns the chondrocytes become larger and increasingly separated by matrix. The progression is proliferation, maturation, hypotrophy, death and lacunar arrangement, followed by the calcification of the matrix. The zones of the epiphyseal cartilage are clearly demarcated. Chondrocyte morphology varies from one zone to the next. This in fact is the method by which the zones are delineated histologically.

MORPHOLOGY OF CHONDROCYTES

Microscopic studies of chondrocytes from various anatomic locations reveals differences in chondrocyte morphology. For the mandibular condyle, obvious morphologic differences occur within the four zones previously described. Blackwood (1966) described two populations of cells present in the resting zone. The superior portion is composed of somewhat oblong and smaller thin cells, while a lower portion, termed the prehypertrophic zone contains larger and more rounded cells. Blackwood felt that these were distinct and separate populations of cell types. In the proliferative zone, chondrocytes of the mandibular condyle undergo mitosis. These cells display highly variable morphological features. Blackwood suggested that cells of this zone mature and serve to maintain the population of mature chondrocytes. Silberman et al., (1973)

described two populations of cells evident in the hypertrophic zone. The first of which is a variety of large, round, pale cells possessing short peripheral processes. A second is described as a darker fibroblastic-like cell with elongated peripheral processes. Silberman also reported the presence of morphologically distinct cell types in the zone of erosion. It is not known if these morphologic differences represent distinct populations of cell types or are merely variations of a single cell type. In later studies, Silberman et al., (1987) utilized immunohistochemistry to demonstrate phenotypic differences in cells of the various zones. Using antibodies to type I collagen, type II collagen, fibronectin, osteonectin, alkaline phosphatase, sialoprotein II, and proteoglycan, he found that the phenotypic expression of the various proteins by chondrocytes of the mandibular condyle altered from one zone to the next.

Chondrocytes of the mandibular condyle, nasal septum, and sphenoocipital synchondrosis were examined in culture by Takigama et al., (1984). Morphologic features were found to vary depending on the tissue of origin. In their study chondrocytes were obtained from the nasal septum, the spheno-occipital synchondrosis, the mandibular condyle, and the costal growth cartilages from the New Zealand white rabbit. After being carefully dissected the cartilages were minced and incubated with 0.1% EDTA in CMF (pH 7.4) for twenty minutes and then with 0.2% trypsin for one hour. Following incubation, the specimens were digested with 0.1% collagenase for the mandibular condyle and 0.2% collagenase for the spheno-occipital synchondrosis, nasal septum, and costal cartilages, for 1.5 hrs. and three hrs. respectively. The resultant cell suspensions were filtered and washed and plated onto culture dishes. The chondrocytes were

photographed on the third day following staining with toluidine blue. Chondrocytes from the costal growth cartilages had an intermediate shape between stellate and polygonal. Nasal septum and spheno-occipital chondrocytes were stellate and the mandibular condylar chondrocytes were polygonal. Takigama also utilized various seeding densities and found this to be another factor affecting cell morphology. Chondrocytes seeded in culture at low density 3×10^5 cells/cm^2 tended to be more stellate in contrast to the polygonal appearance of chondrocytes seeded at high density 8×10^5 cells/cm^2. Seeding density also affected the rates at which cultures achieved confluency. Low density cultures exhibited the slowest growth rate and highest degree of morphologic variation. Takigama recommended 3×10^5 cells/cm^2 for maximum morphologic stability and growth rate.

The morphology of epiphyseal chondrocytes was further examined by Salentijn et al., (1987). They utilized a computer aided three dimensional reconstruction to characterize the morphology of chondrocytes in rabbit epiphyseal growth plates. In their study 7-10 week old New Zealand white rabbits were utilized. Tissues were fixed by profusion with 10% formalin followed by decalcification in 5% formic acid. Block sections were embedded in paraffin and serially sectioned at 10 microns. The sections were stained with hemotoxylin and eosin and marked for proper orientation. Each section was projected and traced through 40-50 serial sections. A total of 431 columns were analyzed and plotted. With the aid of computer reconstruction, the columns were analyzed. Of the 431 columns only 258 progressed the entire length of the growth plate. They demonstrated the presence of a small zone consisting of cells

morphologically distinct from the chondrocyte columns normally described for this tissue. This small cell zone bordered the organized pallisades in the epiphyseal growth plate. They proposed that these small cells act as a source of new cells for the ongoing proliferative process. They also described flattening of cells in the proliferative and upper hypertrophic zones, suggesting alternative functional roles for various cell types. As the growth rate decreased, these investigators noted that chondrocytes became more rounded. These studies indicate that chondrocytes could eventually be classified by morphologic and functional features.

When examining chondrocyte morphology, care must be taken to consider many variables. Silberman et al., (1973) found different methods of fixation to be a factor affecting morphology. In his study Silberman utilized buffered osmium tetrozide 1% and 2%, Gluteraldehyde 2.5% and 5% followed by post fixation with 1% osmiumtetroxide, modified karnovsky medium, simultaneous fixation with gluteraldehyde and osmium tetroxide, freeze substitution using acetone alone and acetone with 1% osmium tetroxide. All specimens were rinsed with phosphate buffer 0.1 m pH 7.4 and dehydrated in a graded series of alcohols and then embedded in Epon 812. They were then sectioned and stained with azure II, basic fusion and alkalinized methylene blue, hemotoxylia and eosin, modified hemotoxylin-safranin and several other stains as well. Silberman found that sections utilizing certain fixation and staining patterns made distinctions visible that were not apparent in others. This may account for variations in descriptions offered by different authors.

Therefore, it is necessary to consider these variables when noting differences from one study to the next.

ISOLATION OF CHONDROCYTES

Chondrocytes to be studied in culture are isolated by various techniques. Isolation techniques often involve the use of proteolytic enzymes, such as trypsin, collagenase, and pronase, to free the cells from their associated matrix. Whole cartilage can be frozen or immediately subjected to sequential digestion to remove matrix components. Liberated chondrocytes are usually recovered by centrifugation after passing through a mesh at the base of digestion chambers (Malemud, 1981). The proteolytic enzymes used to obtain these chondrocytes have pronounced effects on membrane proteins as well as matrix components, (Gertzbein et al., 1977; Weibkin and Meir, 1977; and Glant, 1986). Cell surface antigens consist of a wide range of attachment factors, histocompatibility antigens, proteins involved in the transfer of metabolites across the cell membrane, glycoproteins for cell to cell interactions all of which are susceptible to digestion. The digestion of these surface antigens by proteolytic enzymes could alter biochemical assays involved in their detection. The absence of digested proteins or the reduction in size of remaining proteins would yield false results in migration electrophoresis. As a result, cells must be allowed to recover for some time before they can be analyzed biochemically. The time interval for recently isolated chondrocytes to begin to synthesize a full range of membrane proteins, is called reconstitution. The ability of a cell to reconstitute can affect the results of

any biochemical assay. The range of time allowed for the reconstruction process has been reported from one hour (Mollenhauer, 1982) to twenty four hours (Gant, 1986). This range is highly variable and to date has not been standardized. Therefore, reconstitution time must be kept in mind when examining protein profiles of chondrocytes.

STABILITY OF CHONDROCYTES IN CULTURE

The effects of various seeding densities on the de-differentiation has also been examined (Watt, 1988). De-differentiation is the term given to chondrocytes in culture which begin to express different phenotypes. Normal cartilage matrix consists predominantly of type II collagen, high molecular weight proteoglycans, and hyaluronic acid. When seeded at low densities, articular chondrocytes begin to synthesize type I collagen along with small molecular weight non-aggregating proteoglycans. The degree of de-differentiation is also directly proportional to culture time and the number of cell passages. Watt reported that by twenty one days in culture both high and low density cultures were expressing type I collagen phenotypes. Handly et al., (1978), reported on the composition of matrix components of confluent cultures after twenty days. They found glycosaminoglycans varied in the relative percentage of sulphation isomers. The ratio of chondroitin 6-sulphate to chondroitin 4-sulphate decreased with increasing time in culture. Handley and his coworkers felt that changes in sulphation patterns may be due to extrinsic variables such as culture medium, changes in pH, as well as time in culture. Benya et al., (1982) reported that the de-differentiation process can be controlled or

even reversed when cultures are transferred to agrose gels or to cultures which limit cell proliferation such as spinner cultures.

ANTIGENICITY OF CHONDROCYTES

Chondrocyte membrane proteins have been studied extensively in arthritis research and have been implicated in certain autoimmune diseases such as rheumatoid arthritis. Because chondrocytes are normally surrounded by non-antigenic intact matrix and receive nutrients by a process of diffusion, they are not normally exposed to the immune system. Trauma to the cartilage may expose certain membrane constituents and trigger an immune response. This autoimmunity to chondrocyte antigens has been offered as a partial explanation for the chronic destructive process associated with rheumatoid arthritis. Characterization of potential autoantigens has been extremely valuable in developing diagnostic aids in screening patients for rheumatoid arthritis (Nemeth-Cosoka et al., 1988; T. Glant 1986; Gertzbein et al., 1977; J. Mollenhauer et al., 1988). Membrane antigens have been classified into subsets consisting of species-common and species-specific chondrocyte epitopes and differentiation antigens (Gertzbein, 1977; Glant, 1986). Histocompatability antigens have also been recognized on the chondrocyte membrane (Glant, 1981) and have been implicated in transplant rejection of cartilage as well as other tissues. These antigen subsets were further studied by Glant who compared the antigenicity of bovine, human, and canine articular cartilage. Using antisera to cell membrane, Glant found that when adsorbed against two of the three chondrocyte populations the antisera still had reactivity to the

third population. The antisera activity that remained after adsorption was bound to antigens classified as species-specific. Antisera activity due to species common epitopes was removed by adsorption. In the same study Glant compared antisera activity and noted dissimilar effects of human rib, laryngeal and articular chondrocytes as well as the bovine rib and articular chondrocytes. This finding suggests important antigenic differences between cartilages. From the differences noted by Glant, it can be implied that cartilage from different anatomic locations may also have epitopes present which could be classified as tissue specific antigens.

Chondrocyte antigens have also been described by Burmister et al., (1983). They described the expression of an Ia antigen on the surface of chondrocytes obtained from patients having rheumatoid arthritis. Ia antigen expression has been implicated in other autoimmune diseases such as juvenile diabetes mellitus, lichen ruberplanus, and primary billiary cirrhosis (Jahn et al., 1987). Ia antigens have also been implicated in allograft rejection. The presence of Ia antigen on the chondrocyte membrane indicates 'transformed' (Burnester et al., 1983). Transformed chondrocytes are capable of releasing enzymes into the surrounding matrix. These enzymes actively resorb matrix components. From this information it is possible, in fact likely, that transformed chondrocytes actively contribute to the destruction of cartilage in certain disease states.

Antigenic components of chondrocyte membranes have been valuable in immunohistochemical analysis of cartilage. They have allowed for the localization of common antigenic determinants between different cartilage types as well as demonstrating that not all cartilage is immunologically identical. Antigenicity has been shown to be a factor in autoimmune

diseases and has allowed for the development of tests to diagnose disease processes.

When studying the antigenicity of cartilage it is important to also consider the matrix. To differentiate between membrane bound and matrix antigens, the membrane bound matrix components must be separated during the isolation process from non-bound matrix components. This is commonly done using collagenase and trypsin.

INTERACTION BETWEEN CHONDROCYTES AND THEIR MATRIX

The interactions of chondrocytes with their surrounding matrix involves a complex series of protein regulated reactions. These reactions have been studied in depth by numerous investigators (Silbermann et al., 1987; Weiss et al., 1988; and Hewitt et al., 1981). They are regulated by surface proteins on chondrocyte membranes. Kosher and Church (1975) showed that variations in the the macromolecular composition of extracellular matrix could effect chondrocyte metabolism and phenotypic expression through some sort of feedback inhibition. Von der Mark (1987) has also demonstrated that chondrogenic differentiation could be affected by the composition of the extracellular matrix. These types of studies have led to the isolation and characterization of numerous membrane components.

Isolation of various membrane proteins has been valuable in studying chondrocyte attachments to collagen and other matrix components. Mullenhauer (1984) has characterized a 31Kd protein from the cell membrane of chick sternal chondrocytes. This 31Kd protein has been

called anchorin CII. Using antibodies directed to anchorin CII, Mullenhauer was able to demonstrate the presence of this protein in the cell membrane of chick chondrocytes. He demonstrated on cryostat sections and in cell cultures that the amount of of anchorin CII on the surface of flattened chondrocytes was reduced. Because flattened chondrocytes are devoid of type II collagen they concluded that the attachment of chondrocytes to type II collagen could be inhibited by antibodies prepared to ancohrin CII.

Vaughan et al., (1987) also utilized chick sternal cartilage to isolate and characterize a six armed glycoprotein greater than 10^6 daltons. On reduction this glycoprotein gave rise to sets of doublets at 205/195 Kd and 220/170 Kd. Using antibodies directed to this glycoprotein, Vaughan was able to demonstrate that this molecule was identical to others previously described as the myotendeous antigen (Chiquet and Fambrough (1984) hexabrachion (Erickson and Inglesias, 1984)). This was confirmed by the use of monoclonal antibodies and rotary shadowing techniques. Vaughan et al., (1987) proposed this glycoprotein to be the ideal substrate to connect widely spaced components in the highly hydrated tissues such as cartilage. The six armed glycoprotein is a large molecule with multivalency and could therefore act as a stabilizing component of the cartilage matrix.

Hewitt et al., (1981) described the isolation and characterization of chondronectin from chick sternal chondrocytes. Chondronectin has also been implicated in chondrocyte interaction with matrix components and the binding of collagen substrates. Using immunofluorescent techniques, Hewitt demonstrated that chondronectin was immunologically distinct from fibronectin and laminin. These studies have allowed others to

propose models for additional membrane components to describe interactions of cells with their surrounding matrix. Prehm (1988) proposed the presence of a membrane protein, hyaluronate synthetase. Hyaluronate is a component of the extracellular matrix. It has been implicated in numerous cellular and pathologic functions including migration of mesenchymal cells, invasion of tumor cells and viral transformation of fibroblasts and chondrocytes. Preham (1988) proposed further that if one could inhibit its synthesis at the cell membrane with the use of an antibody directed toward his proposed protein it would be valuable in characterizing its role further. Immunohistochemical analysis has made it possible for investigators to classify molecules as well as establishing a method by which molecules examined in separate studies could be compared for likeness. This eliminates undue nomenclature for the same molecules studied by different investigators.

ANTIGENIC COMPONENTS OF CARTILAGE MATRIX

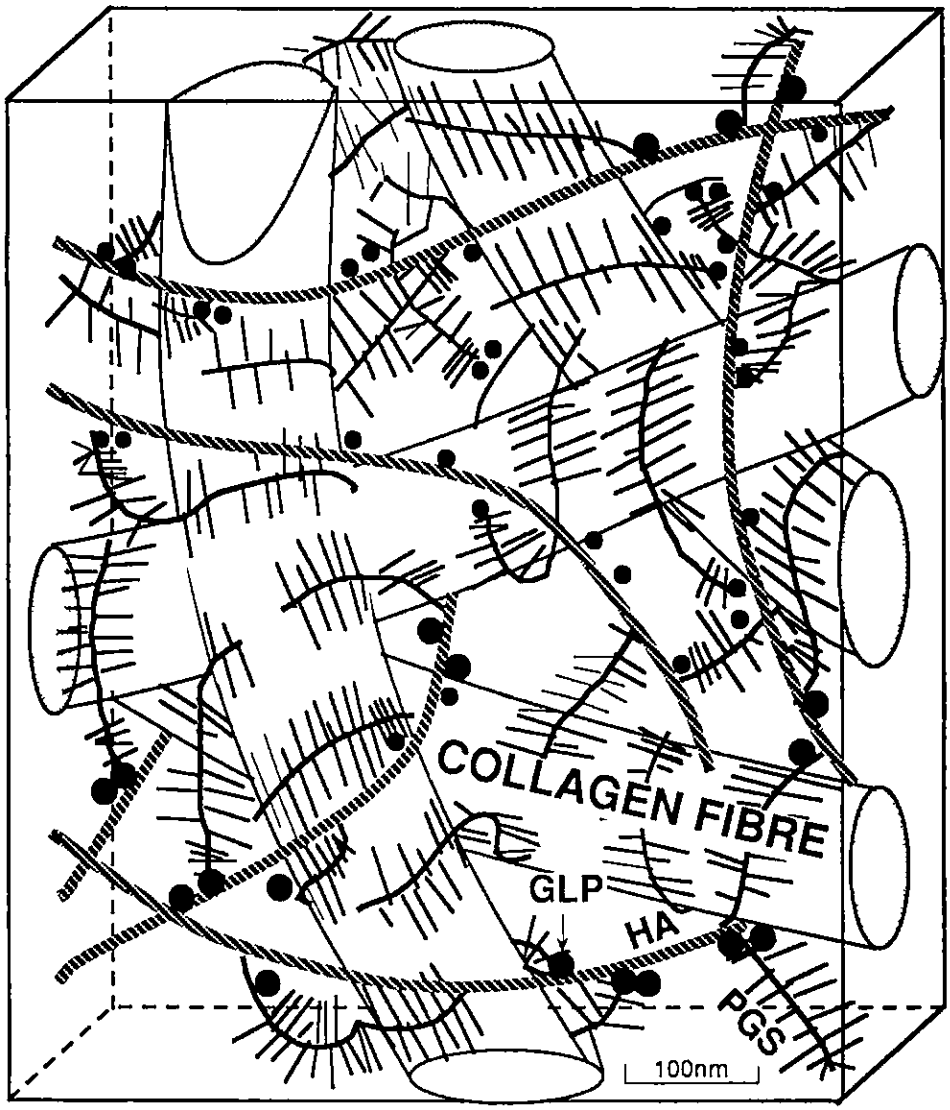
Potential antigenic components of cartilage are present on the cell membrane and in the matrix. Cartilage matrix consists of numerous collagen types and a hyaluronic acid backbone with non-covalently attached proteoglycan monomers (fig. 2). Each proteoglycan monomer consists of a core protein with bound chondroitin sulphate, keratin sulphate, and O-linked and N-linked oligosaccharides. The monomeres are attached to the hyaluronic acid backbone through link proteins (fig. 7). Each of these components have been explored by various investigators to determine there antigenicity. Antibodies raised against these various

components are used to study each components role and location in the intact cartilage matrix.

Type II collagen is the most prominent collagen in cartilage matrix, however numerous minor collagens have also been described (fig. 3). Klienman et al., (1981) in reporting on the role of collagen in adhesion and growth of cells, described the presence of collagen types II and V in freshly isolated chondrocyte cultures, which with increasing time in culture chonrocytes began to produce types I, III, and type I trimer. Vasios et al., (1987) described the presence of type IX collagen in cartilage matrix. Using gold labeled antibodies directed toward this minor collagen, they were able to demonstrate its close association with type II collagen in cartilage matrix. The antigenicity of various collagens and collagen sequences has been utilized to produce antibodies which can be used to study the role of collagen in cartilage matrix. The characterization of auto-antibodies directed to various collagen sequences has been valuable in developing diagnostic screening tools for patient's with certain autoimmune diseases. (Golds et al., 1983; Champion et al., 1983).

Polyclonal and monoclonal antibodies have been raised to various other matrix components. Jenkins et al., (1981) and Couchman et al., (1984) have raised monoclonal antibodies to saturated and unsaturated oligosaccharides of chondroitin sulphate. Carterson et al., (1982) and Fudenburg et al., (1983) monoclonal antibodies have also been raised against intact keratin sulphate, Caterson et al., (1982) and Stevens et al., (1985) the binding regions on hyaluronic acid, Dorfman et al., (1980) and Upholt et al., (1979) various regions on core protein, Glant et al., (1986) and to four separate regions of core protein from human articular cartilage.

FIGURE 3
Schematic Of Cartilage Matrix



Sorrel et al., (1988) other researchers have reported monoclonal antibodies to carbohydrate epitopes of chondroitin 4 sulphate. Ruggiero et al., (1988) and to c-propeptide of chick type II collagen. These studies have demonstrated the utility of using monoclonal antibodies to study specific structural components and to identify their locations and functions. These advanced immunologic methods have greatly enhanced our knowledge concerning the organization of cartilagenous matrix.

Utilizing antibody studies, it has been shown that cartilagenous matrix changes with age. Using four monoclonal antibodies Glant et al., (1986) reported that segments of the core protein and keratin sulphate bound to core protein were not stable throughout life. They reported that the matrix is constantly changing as the age of the subjects increased. Alterations in amino acid sequences of core proteins as well as changes in binding patterns of keratin sulphate altered the reactivity of their antibodies. This suggested that human cartilage contains at least two distinct populations of proteoglycans which may represent the products of different genes or post translational modifications which are age dependent. Hjertquist et al., (1972) had previously described variations in the molecular weight distributions of chondroitin sulphate derived from subjects of different ages. Thoner et al., (1986) also described variations with age in buoyant densities of proteoglycans in CsCl gradients. It becomes apparent from these studies that cartilage is a very complex tissue. The need for standardization of experimental conditions exists. Results of biochemical assays, are influenced by a wide range of variables which must be controlled for if consistent progress is to be made.

GROWTH STUDIES UTILIZING ANTI-CARTILAGE ANTIBODIES

To study the growth process using anticartilage antibodies requires the identification of antigens unique to the cartilage of interest. Brigham (1976) addressed this issue studying bovine cartilage. He examined nasal septal, condylar, and epiphyseal cartilage and Brigham first identified that condylar cartilage contained unique protein bands in acrylimide gels stained with comassie blue. Using polyclonal antibodies raised in rabbits to cartilage extracts Palmer (1978) demonstrated that antibodies could be localized specifically to cartilage. Hans (1981) utilizing a cytotoxic antibody, raised in a similar fashion, directed against the nasal septum, demonstrated statistically significant alterations in midfacial growth. Rats were injected with the septal antibody as they grew. Localization of the antibody to the matrix and cellular components of the nasal septum as demonstrated and cephalometric analysis of the experimental group showed a decrease in snout length. Perih (1986) was able to prepare and react anticartilage antibodies to numerous cellular and matrix antigens, demonstrating that it would be possible to study cartilage with anticartilage antibodies. In line with previous studies, various cartilages will be examined for the presence of unique antigenic determinants for the production of specific anticartiladge antibodies.

STATEMENT OF THESIS

The purpose of the present study is to determine if there are specific proteins unique to the cartilages of the nasal septum, mandibular condyle, and epiphyseal growth plates in the 9-11 day old Sprague Dawley rat. Protein profiles will be identified using SDS polyacrylamide gel electrophoresis and silver stain. Proteins from the cell membranes of chondrocytes of each of the cartilages will be compared from cultured and freshly isolated chondrocytes. The freshly isolated chondrocyte membrane proteins from each of the three cartilages will be compared at two recovery intervals, twelve and twenty four hours. The twelve and twenty four hour reconstitution intervals will be compared to chondrocyte membrane proteins obtained from cultured chondrocytes allowed to reach confluency in 75 cm^2 culture flasks forty three days after isolation. The matrix proteins of the three cartilages will also be compared from both freshly isolated and cultured cartilage. Potential antigenic proteins will be classified as bands of silver stain which are unique to each sample apparent on the acrylimide gels. After the identification of unique bands preliminary screening of antisera previously prepared by Hans and Perih will be tested for reactivity.

The present study will also compare the morphology of cultured chondrocytes from the mandibular condyle, nasal septum, and epiphyseal cartilages at both low and high density. The chondrocytes will be grown on cover slips and photographed for comparison.

This study is in series with others conducted in our laboratory by Brigham (1976), Palmer (1978), Hans (1981), and Perih (1986). The overall objective is to more precisely study the role of cartilage in the growth process, using monospecific antibodies directed against specific cartilages. With the perfection of techniques to produce monoclonal antibodies, the presence of proteins unique to each of the craniofacial cartilages would make this type of study possible.

MATERIALS AND METHODS

OVERVIEW

Chondrocytes were collected from nasal septum, condyle, and epiphyseal cartilage of 9–11 day old Sprague Dawley rats and grown in culture. Confluent cultures were extracted with 4M guanidine for separation of cells and matrix. Solubilized matrix was then dialyzed exhaustively against distilled water, lyophilized, and examined by electrophoresis. Cells were separated from soluble matrix by centrifugation and subsequently homogenized in water. The membrane fraction was then recovered by centrifugation. Cell membranes were solubilized in non-ionic detergent, reduced with diotiotreatol, and subjected to electrophoresis for comparison of protein patterns. The cartilage of the mandibular condyle, nasal septum and epiphyseal plates of the femur and humerous were compared to each other for the presence of unique staining patterns for both the matrix and the cell membrane fraction. Comparisons of membrane fractions were made at two different recovery periods, twelve and twenty four hours. Chondrocytes from the above cultures were also grown on cover slips and examined for morphologic and temporal differences.

CELL ISOLATION AND CULTURING

Fresh cells were isolated from extirpated nasal septum, mandibular condyle and epiphyseal cartilages of the femur and humerous of 9-11 day old Sprague Dawley rats. The cartilages were thoroughly cleaned, minced

and placed in a digestion chamber (courtesy of Dr. Charles Malemud CWRU). In the chamber they were reacted with 0.1% trypsin in phosphate buffed saline (PBS) at pH 7.2 for thirty minutes at 37° C and subsequently for three consecutive 45 minute intervals in 0.1% collagenase in PBS until the cartilage was completely digested. Chondrocytes freed from whole tissue passed through a nylon mesh with a pore size of 62 microns at the base of the digestion chamber and were recovered by centrifugation at 1700 g for 2 minutes. Collagenase supernatant was collected and frozen for later use in protein separation (see section V) and for comparison to other samples. Collected chondrocytes were washed and centrifuged three consecutive times with PBS solution to remove collagenase. Isolated chondrocytes were then resuspended in Hams F-12 nutrient medium containing 10% fetal calf serum (FCS), penicillin (100gm/ml) streptomycin (10gm/ml), and Amphotericin B(2mg/ml) and inoculated to T-30 culture flasks. Cultures were maintained at pH 7.2 with 5% CO_2 at 37° C and fed twice weekly with increasing volumes of F-12 medium until they reached confluency. Some primary cultures were harvested with trypsin and frozen in dimethylsulfoxide (DMSO) for later use in protein separation (see section V) and for comparison to other samples. Others were subsequently subcultured to larger 75 cm^2 Corning culture flasks to increase the quantity of available cells (fig. 7).

HARVESTING CELLS AND MATRIX

Confluent cultures were washed three times with (PBS) to remove adherent serum proteins used to supplement the Hams F-12 media. Washed cultures were then subjected to 4M guanidine HCl, and cells were

then scrapped from culture flasks with a rubber policeman to free attached cells. The guanidine solution was allowed to react for 15 minutes to disassociate attached cells and solubilize the extracellular matrix. Harvested cells were collected by centrifugation at 27,000 g, 5° C, for 15 minutes. The cell pellet was centrifuged and resuspended for two consecutive washes in 4M guanidine. After each suspension the solubilized matrix fraction was decanted and saved for dialysis. The resulting cell pellet was suspended in phosphate buffer solution (PBS) to wash, centrifuged and frozen for later use in protein separation (see section V) and for comparison to other samples.

ISOLATION OF CELL MEMBRANE PROTEINS

Collected cells were suspended in double distilled water and homogenized with a glass Teflon homogenizer to liberate intercellular debris. The suspension was then centrifuged at 27,000 g, 5° C, for 5 minutes and the supernatent poured off. The resultant membrane pellet was resuspended in extraction buffer containing the following: 150 mM- NaCl, 50 mM - hepes pH 7.4, 1 mM EDTA, aprotinin 100 kiv/ml, PMSF 2 mM , Ninodet P 40 1%, and allowed to incubate at 5° C for 30 minutes. The resultant suspension was then centrifuged at 27000g for 5 minutes and the supernatant collected for analysis by electrophorisis.

ISOLATION OF MATRIX PROTEINS

The guanidine supernatant from section III for each of the condyle, nasal septum and epiphyseal cultures was collected and dialized exhaustively against 6 volumes (50 L each) of distilled water containing 0.15 M acetic

acid for 96 hours, frozen with dry ice and ethanol and lyopholized. Resultant samples were reduced and analyzed by sodium dodicyl sulphate (SDS) polyacrylimide gel electrophoresis. Samples were compared to each other and to membrane fractions. Matrix fractions were obtained from whole tissue as well as from culture. Cultured cartilage matrix was compared to whole matrix tissue and examined for differences between in vitro and in vivo matrix.

ELECTROPHORESIS

SDS acrylimide slab gel electrophoresis (16 x 20 cm) was used to separate proteins from the above samples (modified from Neville et al., J. Biochemistry (20) 6328 - 6334 (1971). All samples were reduced with 10mM dithiotreatol to pH 6.1 (see appendix I for details).

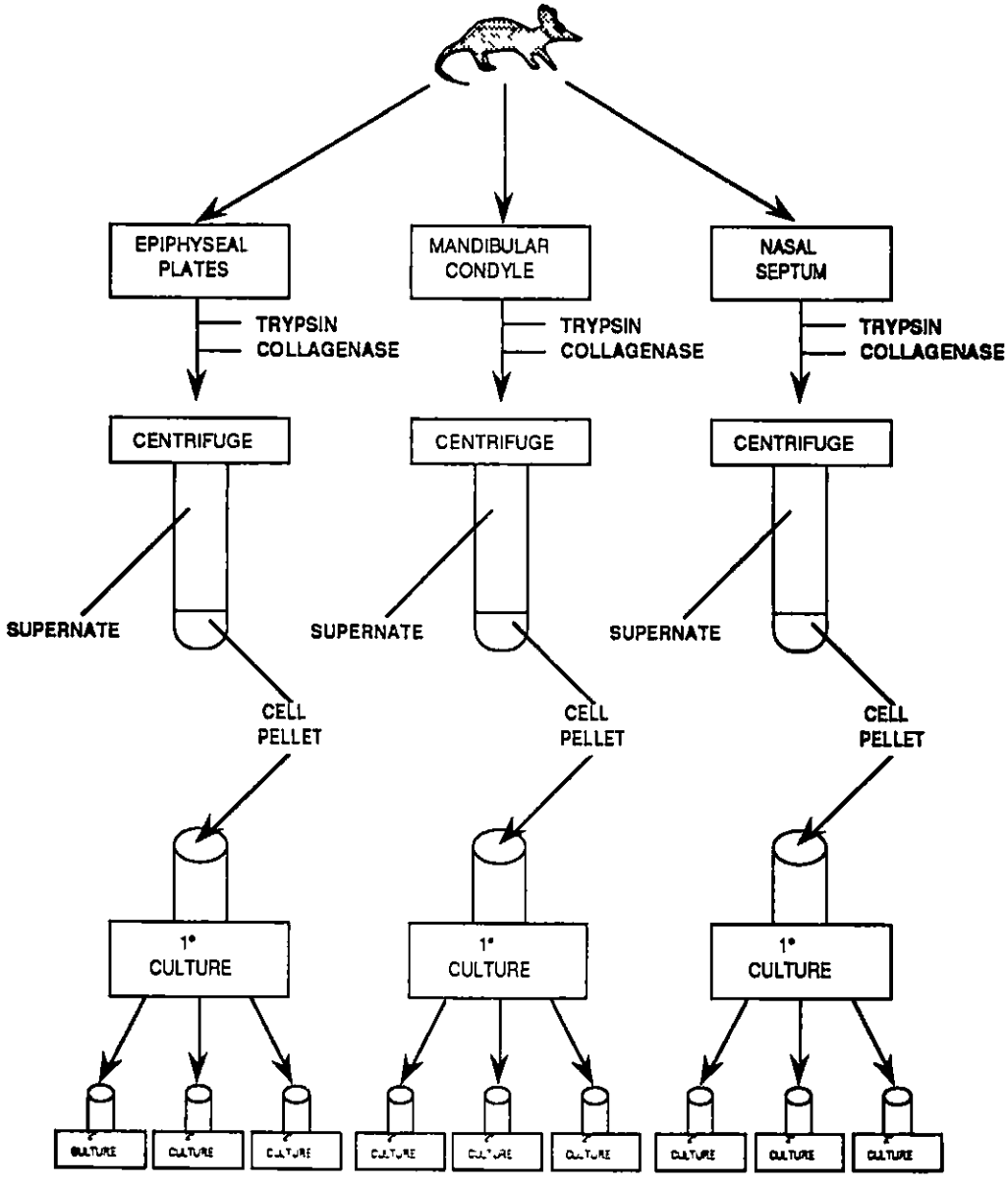
STAINING

Silver stain was utilized to identify protein bands on electrophoretic gels. This stain has been reported to be 100 times more sensitive than commassie blue. It is able to identify protein at a concentration of .07ng/mm^2 for bovine serum. [modified from Oakly, B.R. and N.R. Morris. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem. 105: 361 - 363. 1980 (see appendix II for details)].

MORPHOLOGICAL ANALYSIS OF CHONDROCYTES

Chondrocytes from the mandibular condyle, nasal septum, and epiphyseal cartilages were isolate by the previously described method and inoculated

FIGURE 4
Cell Isolation And Culturing



onto petri dishes containing cover slips. The petri dishes were placed in a air tight chamber with 5% CO_2 at 37° C. Cultures were fed three times a week with increasing volumes of Hams F-12 culture media. Cover slips were microscopically examined at two, six, and twelve days for a comparison of chondrocyte morphology. Cultures were seeded at either low or high density for comparison of morphologic features.

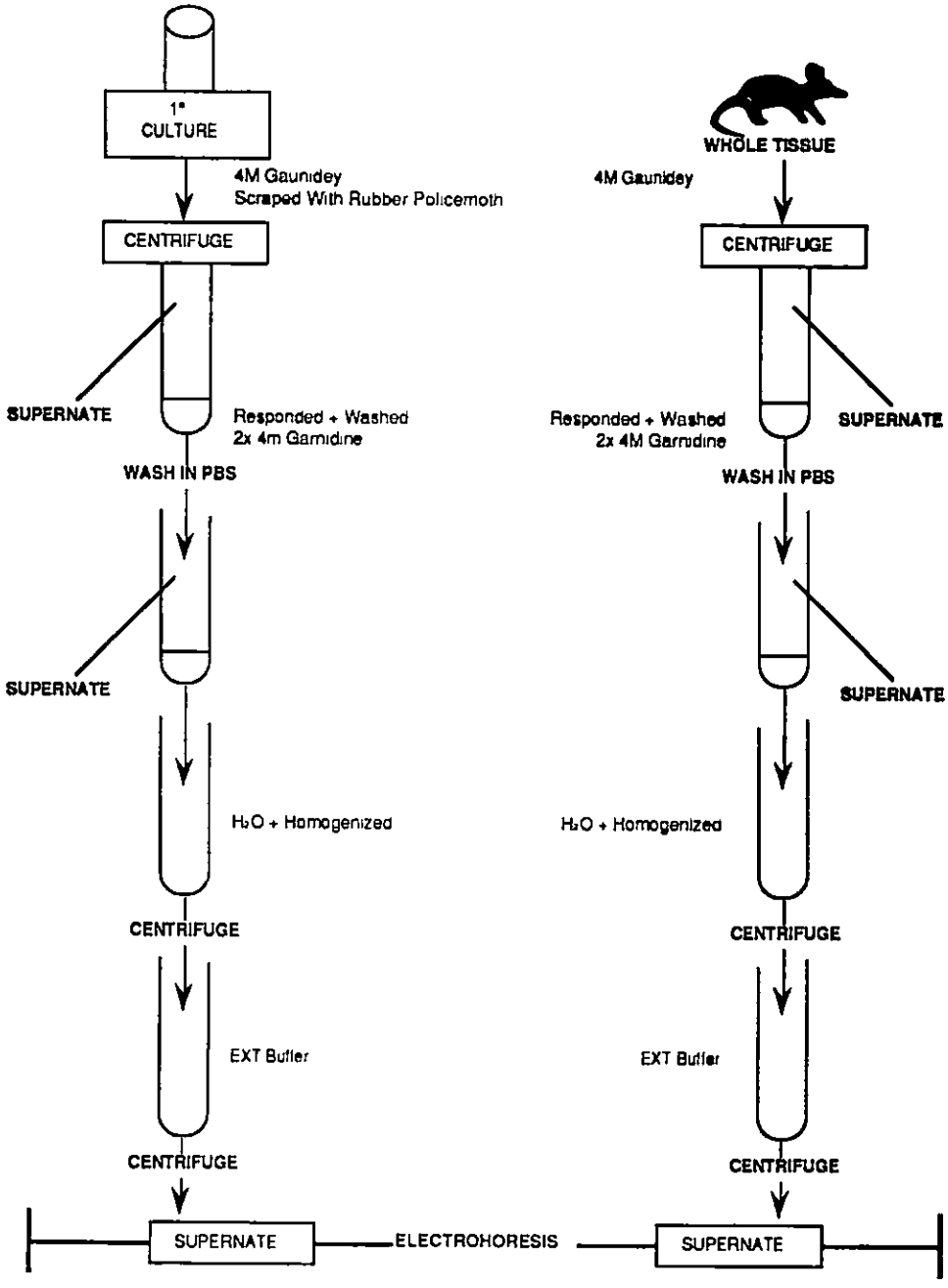
COMPARISON OF CULTURED CHONDROCYTES TO FRESHLY ISOLATED CHONDROCYTES

Fresh chondrocytes isolated from eleven day old sprague dawley rats were used to compare in vivo membrane proteins to those derived from cultured chondrocytes. Chondrocytes were isolated (as previously described) and allowed to recover for either twelve or twenty four hours. Cells were grown in Hams F-12 culture media supplemented with 10% fetal calf serum and maintained at 37° C at pH 7.2 with 5% CO_2. Sample numbers were estimated using a bright light hemocytometer. After incubating for twelve or twenty four hours respectively, cells were washed three times with phosphate buffer solution to remove non-specific bound serum proteins and harvested with a rubber policeman, collected, and centrifuged at 27,000g for 5 minutes, homogenized in double distilled water and respun. The resulting pellet was suspended in extraction buffer for thirty minutes at 5° C., centrifuged and the supernatant, and later evaluated by electrophoresis (see section V).

4M GUANIDINE HCL EXTRACTION OF WHOLE CARTILAGE MATRIX

Cartilage obtained by gross dissection from eleven day old sprague dawley rats, from the mandibular condyle, nasal septum, and epiphyseal

FIGURE 5
Extraction Of Chondrocyte Membrane Proteins



cartilages, as minced and reacted with guanidine HCl for twenty four hours. The resultant suspension was centrifuged at 27,000g for 5 minutes and the supernatant collected, dialyzed exhaustively, and lyophilized. The matrix from the mandibular condyle, nasal septum, and epiphyseal plates was then reduced by beta mercaptoethanol and later examined by electrophoresis.

CsCl SEPARATION DIGESTED & UNDIGESTED CARTILAGE MATRIX

Whole matrix was separated by CsCl density gradient centrifugation in both digested and undigested forms. This system is used to separate cartilage matrix into sections based on molecular weight. The separation of the cartilage matrix into distinct molecular weight ranges makes later immuno assay tests more easily distinguishable. Digests were performed with chondroitinase ABC for four hours and gradients spun at 5° C for twelve hours at 34,000g. Gradients were divided into four sections termed D1 through D4 and prepared for dot blots with antibodies previously prepared by Hans and Perih. Dot blotting is performed to illuminate reactivity of the previously prepared antibodies to individual sections. After reactivity to an individual section has been determined, that section can be separated by electrophoresis and western transferred onto nitrocellulose paper. This will allow for the identification of the precise protein to which the antibodies are reacting.

ANTIBODY PREPARATION

Antibodies were previously prepared in male white New Zealand rabbits by injecting anesthetized animals in the region of the popliteal lymph

nodes with 1 mg of cartilage antigen in a 0.5 ml of a 1:1 mixture of phosphate buffered saline (PBS) and complete Freund's adjuvant. Rabbits were boosted monthly. Serum was collected by intracardial puncture and assessed for antibody titer with indirect immunofluorescence (Hans, 1981; Perih, 1987). IgG was isolated from pooled sera by ammonium sulphate precipitation. The precipitate was then resuspended in distilled water and dialyzed twenty four hours in two ten liter volumes of 0.01M PBS containing 0.03NaCl at pH 7.0. Dialysates were then centrifuged for 45 minutes at 30,000 x g to remove insoluble material and chromatographed under the same conditions on DE23 diethylaminoetheyl cellulose columns. The IgG fractions were recovered in a single peek elution as previously described (Palmer, 1978; Hans, 1988).

DOT BLOTTING OF CsCl GRADIENTS

One dot of each gradient D1 through D4 were placed on nitrocellulous paper and reacted for thirty minutes with anti-condylar antibody previously prepared by Perih, (1987) washed with PBS and counterstained with anti-rabbit florescene labeled goat anti-rabbit IgG Isothiocyanate.

RESULTS

COMPARISON OF MEMBRANE BOUND PROTEINS

Membrane proteins were compared among chondrocytes obtained under the following conditions:

1. Fresh cells allowed to recover for twelve hours in culture.
2. Fresh cells allowed to recover for twenty four hours in culture.
3. Cultured cells
4. Confluent cultured cells frozen in dimethylsuloxide (DMSO), thawed and allowed to recover in culture for one hour.

COMPARISON OF FRESH CELLS FROM THE TWELVE HOUR SAMPLES TO THE TWENTY FOUR HOUR SAMPLES

Analysis of chondrocyte membrane proteins of the above samples demonstrated that chondrocytes dedifferentiate in a short period of time in culture. Protein profiles of chondrocytes from the twelve hour recovery period (A) contain at least three major protein bands which are decreased or absent in the twenty four hour (B), cultured (C), and frozen (D) samples. This was true for all three tissues examined. The mandibular condylar chondrocytes demonstrated a reduction in an additional band as well. Protein bands present in the 100,000 dalton, 70,000 dalton and the 25,000 dalton range for the mandibular condyle, nasal septum and epiphyseal chondrocytes were decreased or absent from the twenty four hour and cultured samples, as well as the frozen samples for the nasal septum and

epiphyseal chondrocytes. A 122,000 dalton protein was absent from the twenty four hour and cultured samples of the mandibular condyle (figs. 7, 8, 9).

COMPARISON OF MANDIBULAR CONDYLE, NASAL SEPTUM, AND EPIPHYSEAL CARTILAGE MEMBRANE PROTEIN PROFILES

When comparing the protein profiles for chondrocytes within each group (mandibular condyle, nasal septum, and epiphyseal cartilages) they were found to be remarkably similar in all respects. No major differences were detected for membrane proteins with the methods employed in this study.

COMPARISON OF EXTRACELLULAR MATRIX PROTEINS IN VITRO MATRIX

Extracellular matrix proteins produced by confluent cultures were compared by eletrophoresis. Marked differences in protein profiles for the mandibular condyle, nasal septum, and epiphyseal extracellular matrix were observed. Unique proteins were present in each sample. Each profile was distinctly different from the other two. When it was observed that a protein band of the same molecular weight, missing from one sample was still present on the remaining two, it was not considered to be unique. With this in mind, at least two protein bands were unique to each sample. For the extracellular matrix of the mandibular condyle, unique bands were present at 38,000 and 43,000 daltons respectively. For the nasal septum, unique protein bands were present at 40,000 and 50,000 daltons. For the epiphyseal cultured extracellular matrix, unique bands were observed at 32,000 and 78,000 daltons respectively (fig. 11). Each of these unique proteins represents a potential antigenic determinant which may be used

for the production of tissue specific antibody to study cartilage. All samples contained only collagenous and minor matrix proteins. None of the samples contained the proteoglycan fragments due to their large molecular weight. In order for proteoglycan fragments to be examined in the 7% acrylamide gels, they must first be digested. There were no chondroitinase ABC digests performed on any of the matrix samples.

IN VITRO MATRIX

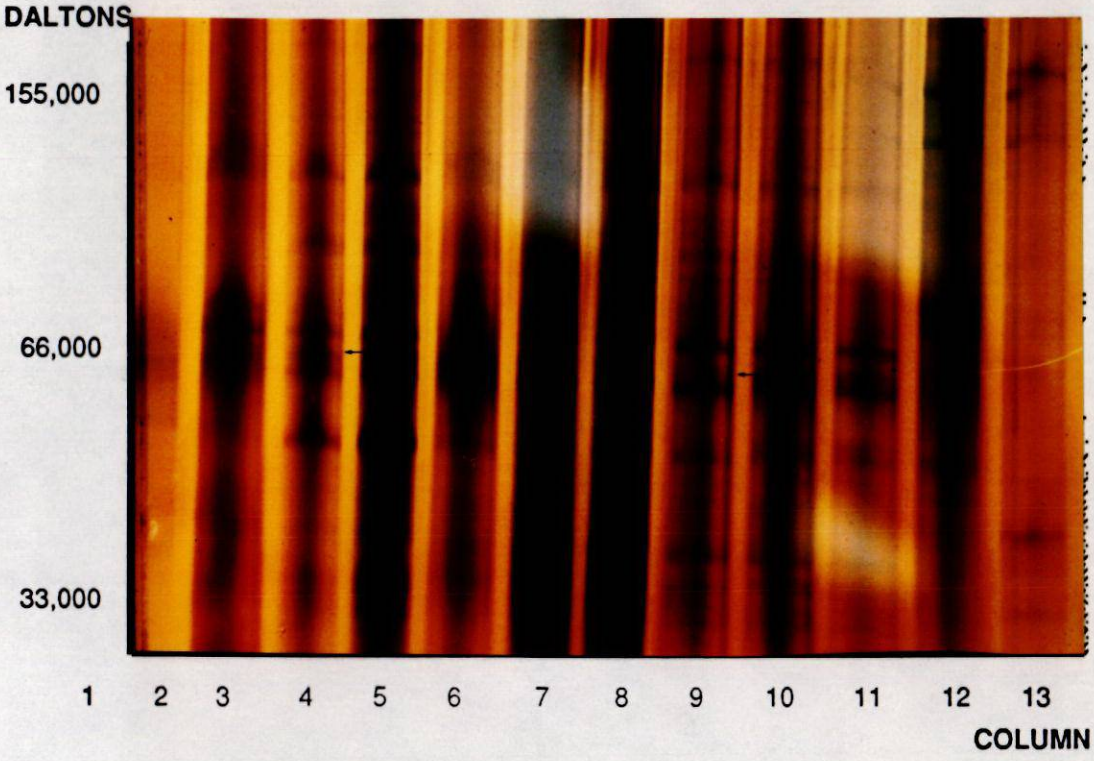
When comparing cultured whole tissue extracellular matrix it becomes very apparent that cultured in vitro matrix is not a true representation of in vivo matrix. The entire protein profile of the mandibular condyle, nasal septal, and epiphyseal extracellular matrix has been transformed. There are relatively few protein bands that remain unchanged. Most of the high molecular weight protein bands are completely absent from the cultured extracellular matrix, possibly due to some digestion by proteolytic enzymes which may have been present and uncontrolled for in this study. Numerous smaller molecular weight proteins are also absent. Proteins in the 30,000, 66,000 and 135,000 dalton range remain unchanged (fig. 10). When comparing profiles from the mandibular condyle, nasal septum, and epiphyseal extracellular matrix from freshly isolated tissue, there were numerous unique protein bands present in each sample. The epiphyseal matrix, proteins in the 20,000 and 35,000 dalton range. The nasal septum, unique bands appear to be present at the 20,000 and 150,000 dalton range. The mandibular condyle appeared to have unique bands present in the 60,000 dalton range (figs. 6, 10).

FIGURE 6
Comparison Of Cultured To Uncultered Matrix



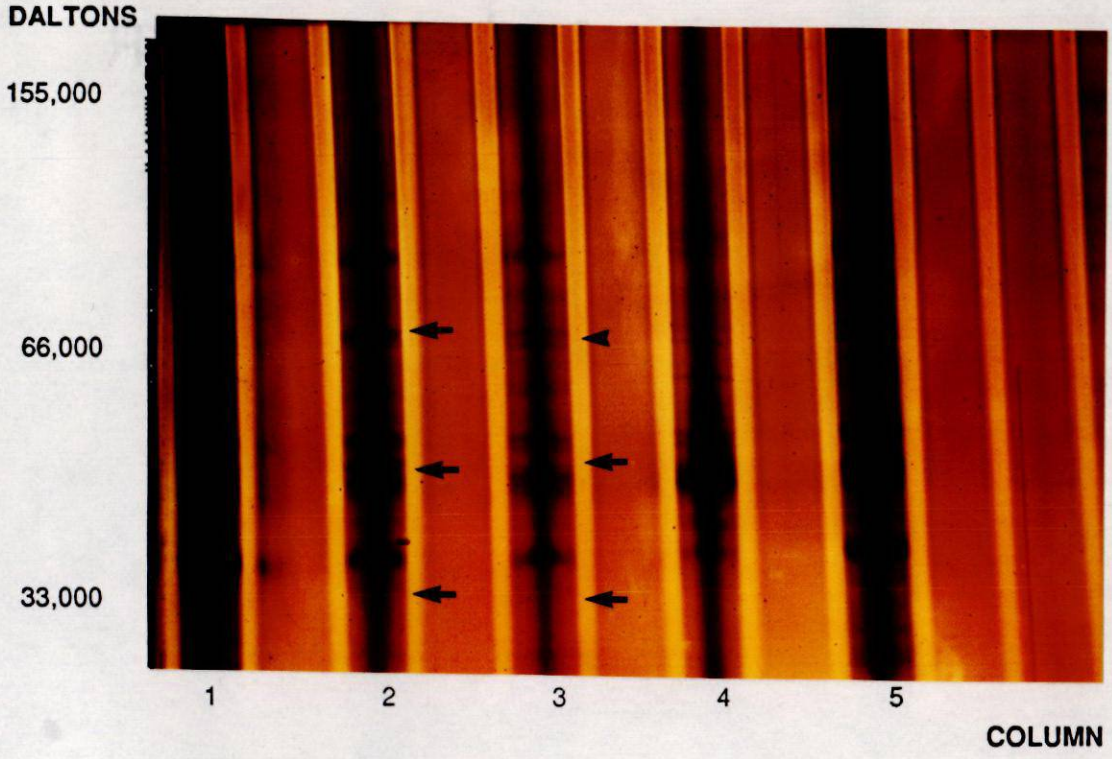
1. Serum
2. Albumin
3. RNA Polymerase
4. CC Not Cultured
5. RNS Not Cultured
6. EPI Not Cultured
7. CC Cultured
8. CC Not Cultured
9. RNS Cultured
10. RNS Not Cultured
11. EPI Cultured
12. EPI Not Cultured
13. Blank
14. Dalton Marker
15. Serum

FIGURE 7
Comparison Of Membrane Proteins From 12 And 24 Hour Recovery Periods



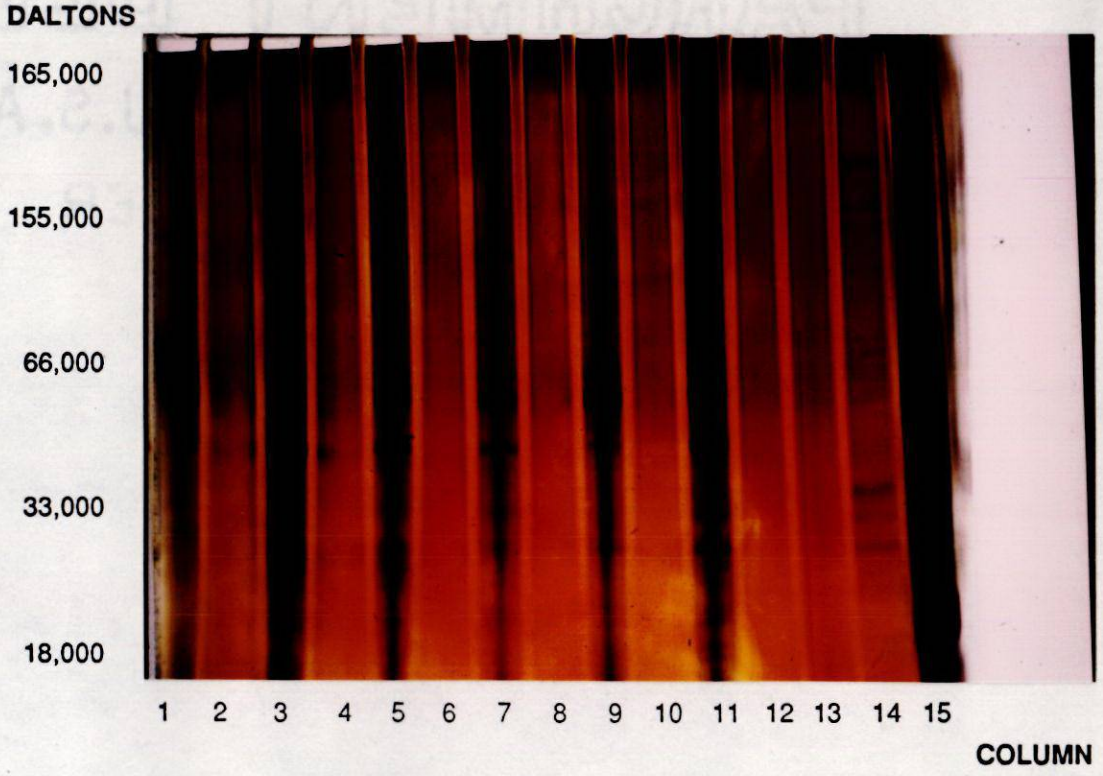
1. Trypsin
2. RNA Polymerase
3. FCS
4. EPI Fresh Cells (12 Hr.)
5. CC Fresh Cells (12 Hr.)
6. RNS Fresh Cells (12 Hr.)
7. EPI Frozen Cells (12 Hr.)
8. RNS Frozen
9. CC Fresh (24 Hr.)
10. EPI Fresh (24 Hr.)
11. RNS Fresh (24 Hr.)
12. CC Cultured
13. EXT Buffer
14. Marker
15. Rinder

FIGURE 8
Comparison Of Membrane Proteins From 12 And 24 Hour Recovery Periods



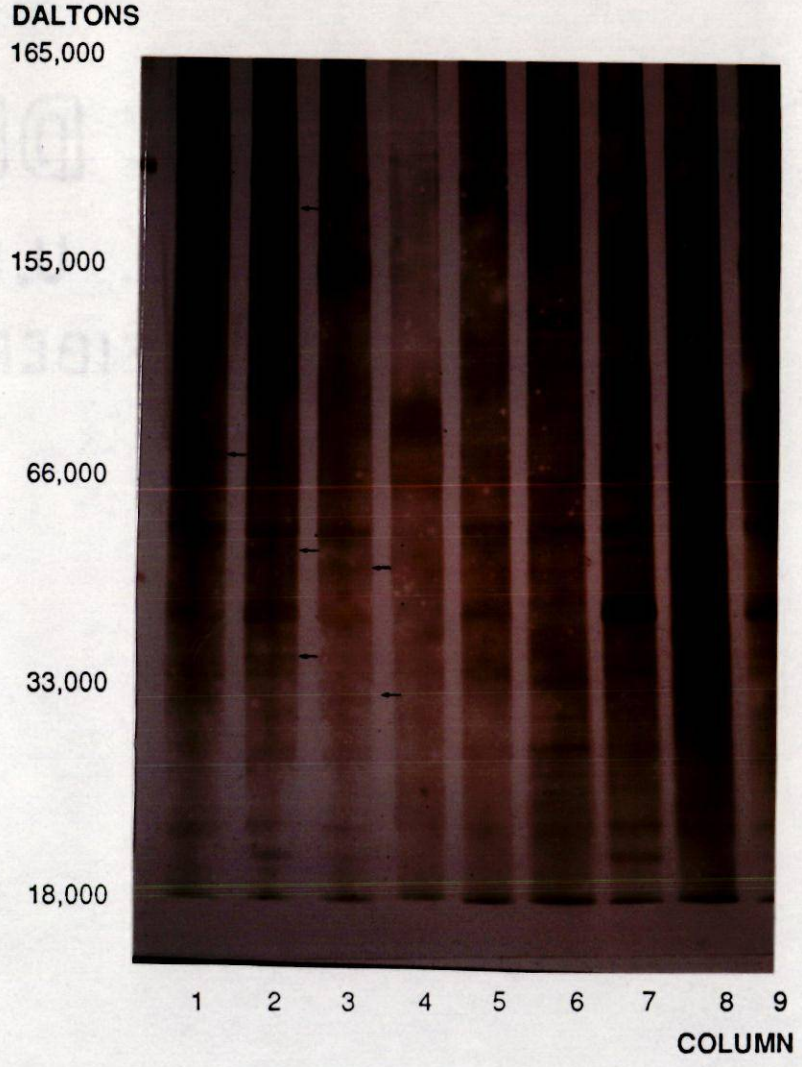
1. EPI Fresh (12 Hr.)
2. RNS Fresh (12 Hr.)
3. RNS Fresh (24 Hr.)
4. CC Fresh (24 Hr.)
5. EPI Fresh (24 hr.)

FIGURE 9
Comparison Of Membrane Proteins From 12 And 24 Hour Recovery Periods



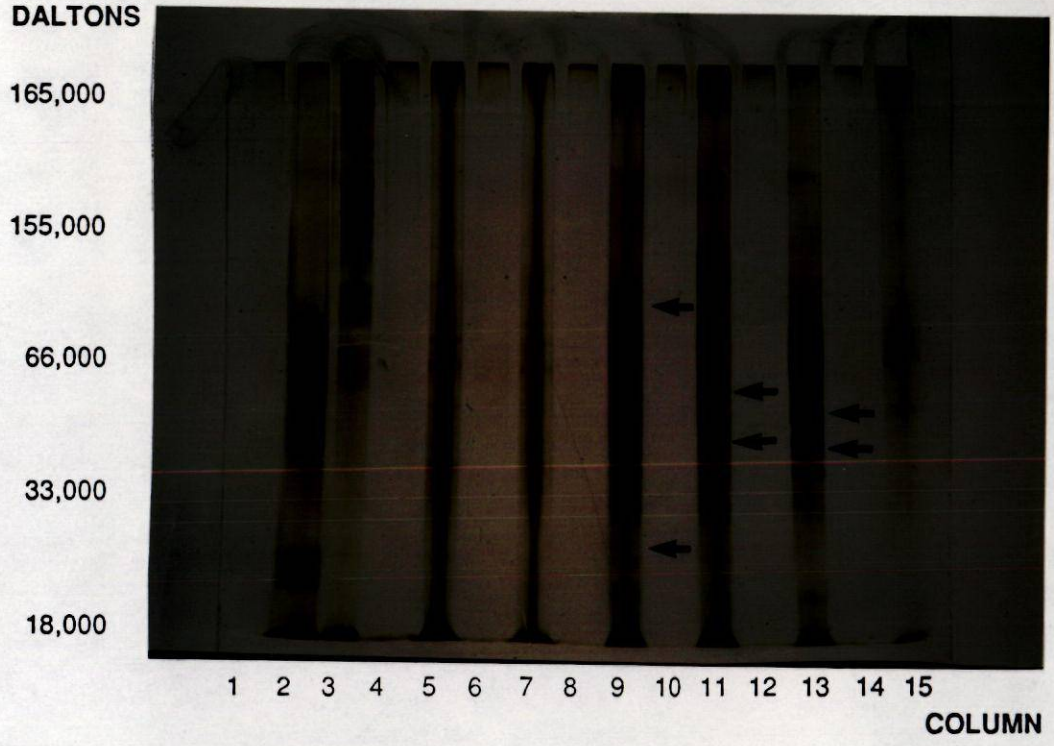
1. CC Fresh (12 Hr.)
2. Blank
3. EPI Fresh (12 Hr.)
4. Blank
5. RNS Fresh (12 Hr.)
6. Blank
7. RNS Fresh (24 Hr.)
8. Blank
9. CC (24 Hr.)
10. Blank
11. EPI (24 Hr.)
12. Blank
13. EXT Buffer
14. RNA Polymerase
15. Dalton Marker

FIGURE 10
Comparison Of Mandibular Condyle, Nasal Septum, And Epithyseal Uncultured Matrix



1. CC Not Cultured
2. RNS Not Cultured
3. EPI Not Cultured
4. CC Cultured
5. CC Not Cultured
6. RNS Cultured
7. RNS Not Cultured
8. EPI Cultured
9. EPI Not Cultured

FIGURE 11
Comparison Of Cultured Matrix



1. RNA Polymerase
2. Dalton Marker
3. Fetal Calf Serum
4. Blank
5. EPI Frozen Cells Membrane
6. Blank
7. RNS Cell Membrane
8. Blank
9. EPI Matrix
10. Blank
11. RNS Matrix
12. Blank
13. CC Matrix
14. Blank
15. Blank

MORPHOLOGIC COMPARISON OF CULTURED CHONDROCYTES

High Density

Chondrocytes seeded at high density ($4x10^6$ cell/cc^3) and allowed to reach confluency assume morphologic features which make them recognizable and distinct based on their tissue of origin.

MANDIBULAR CONDYLE

Chondrocytes derived from the mandibular condyle assume a fibroblastic appearance. They are long spindle shaped cells which become organized in a half staggered interlocking fashion to form large sheets. They possess extensive perpheral processes at one end which extends in the direction of the linear arrangement. At the opposite pole there appears to be two shorter peripheral processes that adjoin two other cells (fig. 12). The cells are of uniform size and morphologic variations are only apparent in areas of intersection of the linear pallasades (fig. 13).

NASAL SEPTUM

Chondrocytes derived from the nasal septum arrange themselves in dense homogeneous sheets at confluence. Each cell remaining separate from neighboring cells by a uniform extracellular space. The cells do not posses peripheral processes and are generally rounded in shape. Some septal Chondrocytes are flattened on one or more sides were they contact neighboring cells. Morphologic variations are present as subtle variations in size and border. For the most part nasal septum chondrocytes are homogeneous when seeded at high density (fig. 14).

EPIPHYSEAL CHONDROCYTES

Chondrocytes of the epiphyseal cartilage display the largest degree of morphologic variation at high density. The cells are separated by varying amounts of extracellular space with no apparent organization. Some chondrocytes possess peripheral processes extending in various directions making cell contact with adjacent cells. The processes are of variable size and shape. Some chondrocytes lack peripheral processes all together, these cells vary from a rounded to an oblong morphology. The size of the cells is also highly variable. Cells possessing peripheral processes are generally triangular in shape with two shorter processes at their base and a longer more extensive process at the apex, extending for variable distances through the extracellular space (fig 15).

CHONDROCYTES MORPHOLOGY LOW DENSITY

At low density, chondrocytes of the mandibular condyle, nasal septum, and epiphyseal cartilage display high degrees of morphologic variation. It was not determined by this study whether these variations represented different cell types, such as fibroblasts or epithylial cells, or merely different degrees of cytoplasmic projections, by cells attempting to reach confluency in culture.

MANDIBULAR CONDYLE

Chondrocytes of the mandibular condyle at low density present several morphologic variations. The most typical, are the elongated fibroblastic appearing cells with extensive peripheral processes. The processes extend great distances apparently till they make cell to cell contact. These

processes extend only from one end of the cell, some contain more then one process or branching of single processes (fig. 16). Also present in low density cultures of mandibular chondrocytes, are much smaller cells which lack extensive peripheral processes. These cells are much less numerous and are not visible when cultures reach confluency (fig. 17). An intermediate cell morphology is also present, these cells have peripheral processes but do not have the elongated fibroblastic appearance (fig. 18).

NASAL SEPTUM

Chondrocytes of the nasal septum seeded at low density also present morphologic variations. The most prominent cell morphology is a triangular shaped cell. These cells lack extensive peripheral processes, have centrally located nuclei, and granular cytoplasm (fig. 19). Very large cells are also present at low density. These cells resemble the smaller triangular shaped cells except for their large size. They appear to spread over large distances until they make cell contact. As the density of the cultures increases, the size of these cells appears to decrease until at confluency these cells are no longer distinguishable (fig. 20). A third morphologic variation resembles the fibroblastic appearance of the condylar chondrocytes. They posses long processes at one end of the cell and shorter less extensive processes at the opposite pole (fig. 21).

EPIPHYSEAL

Chondrocytes of the epiphyseal cartilage present the highest degree of morphologic variation at low density. both size and shape are extremely

variable from one cell to the next. In contrast to chondrocytes of the nasal septum and the mandibular condyle, epiphyseal chondrocytes have peripheral processes that extend from both ends of the cells as well as midpoints along the cell body (fig. 22). As cultures reach confluency morphologic differences remain present.

PRELIMINARY SCREENING OF ANTISERA TO WHOLE CONDYLE

Dot blots were preformed on both digested and undigested fractions D1 through D4 from the CsCl gradient separations. The separation was preformed on condylar whole matrix only. After separation fractions were screened with antisera previously prepared and cross reacted with anti rabbit immunoperoxidase for staining purposes fractions D2 and D3 showed slight reactivity in the undigested samples. D2 and D3 fractions in the digested samples, 4 hour ChABC digests, showed no reactivity to the antisera which was screened.

FIGURE 12

Photograph Of Mandibular Condyle Chondrocytes

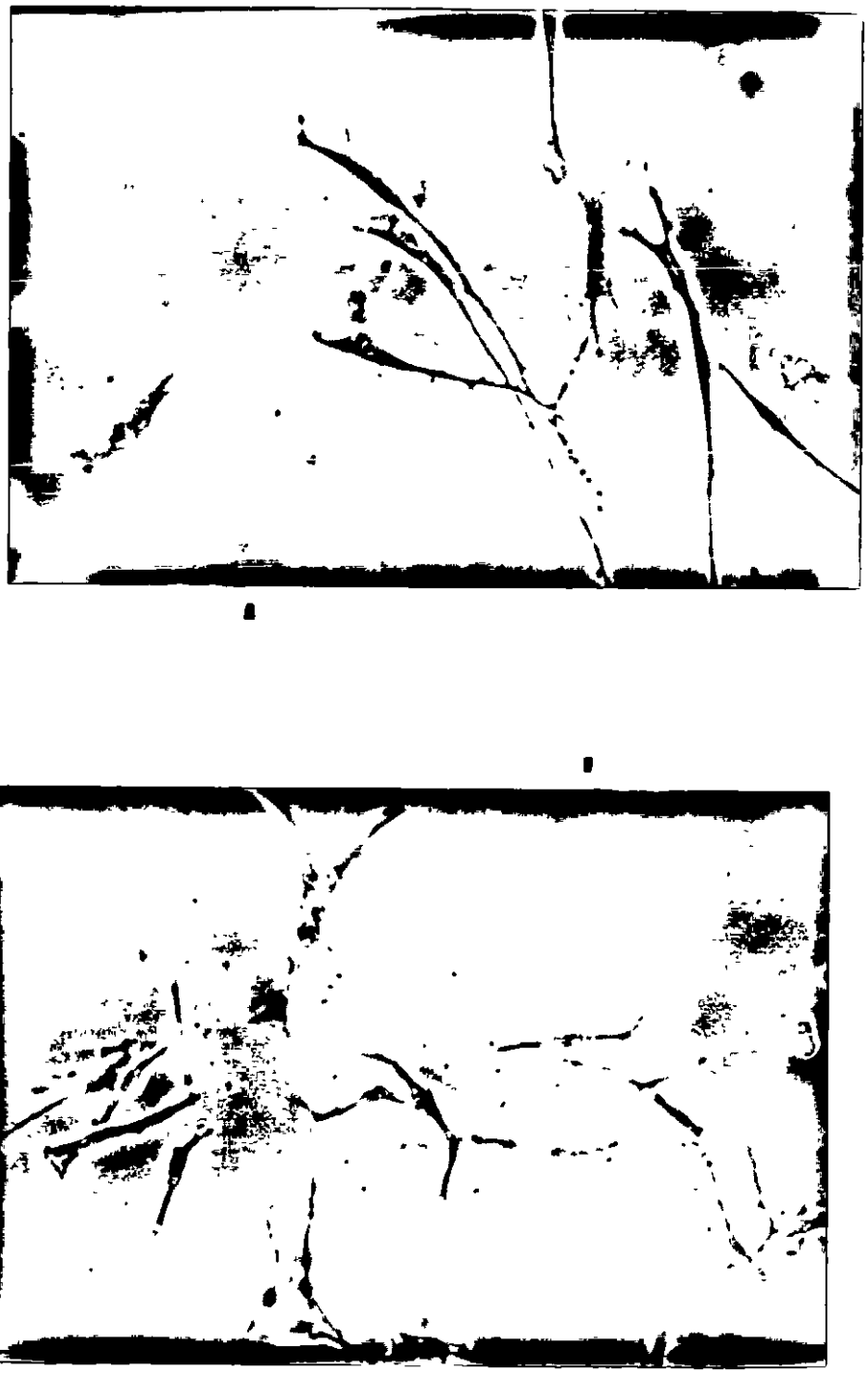


FIGURE 13

Confluent Cultures



FIGURE 14

Photograph Of Septal Chondrocytes Confluent

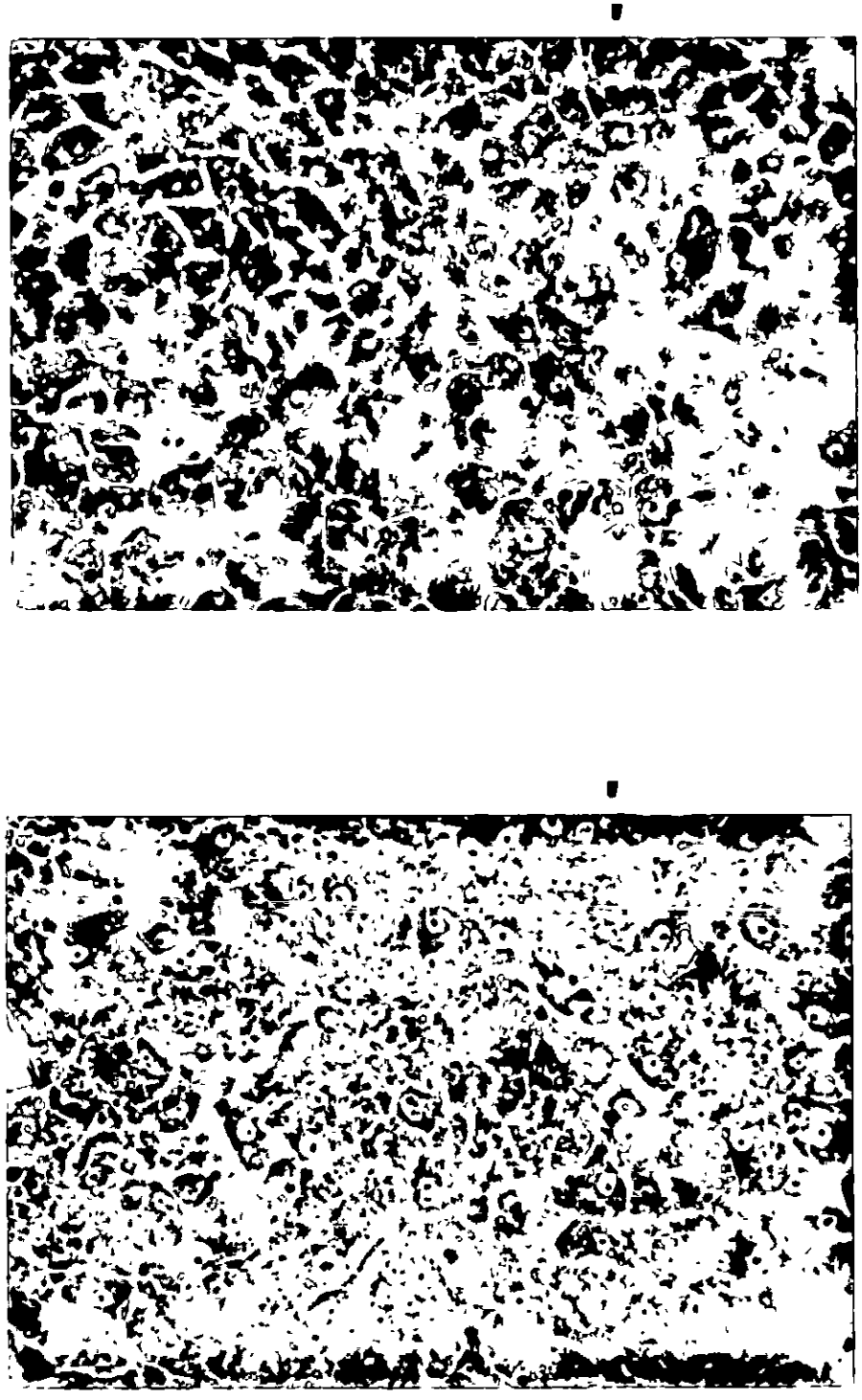


FIGURE 15

Photograph Of Epiphyseal Chondrocytes

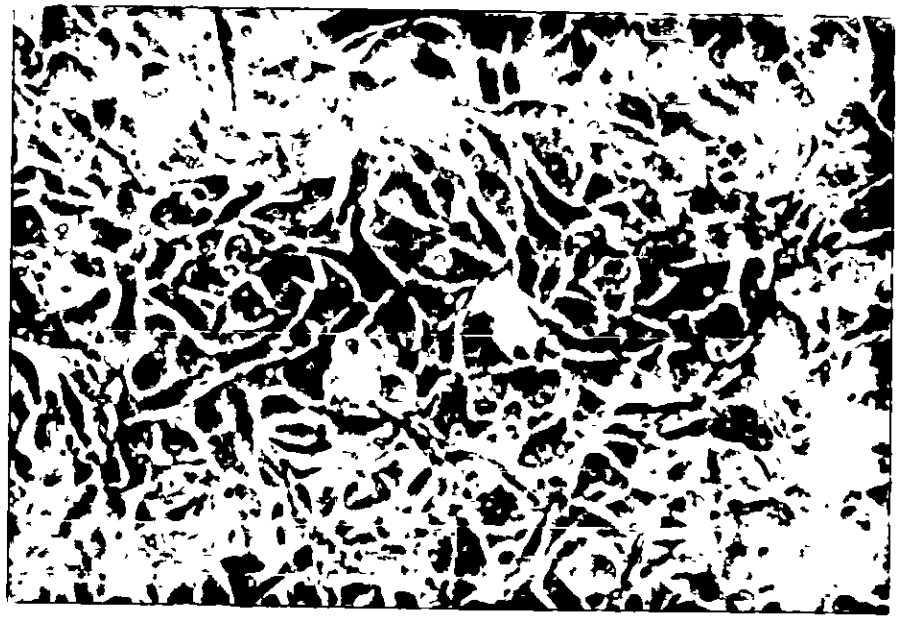


FIGURE 16

Photograph Of Mandibular Condyle Chondrocytes

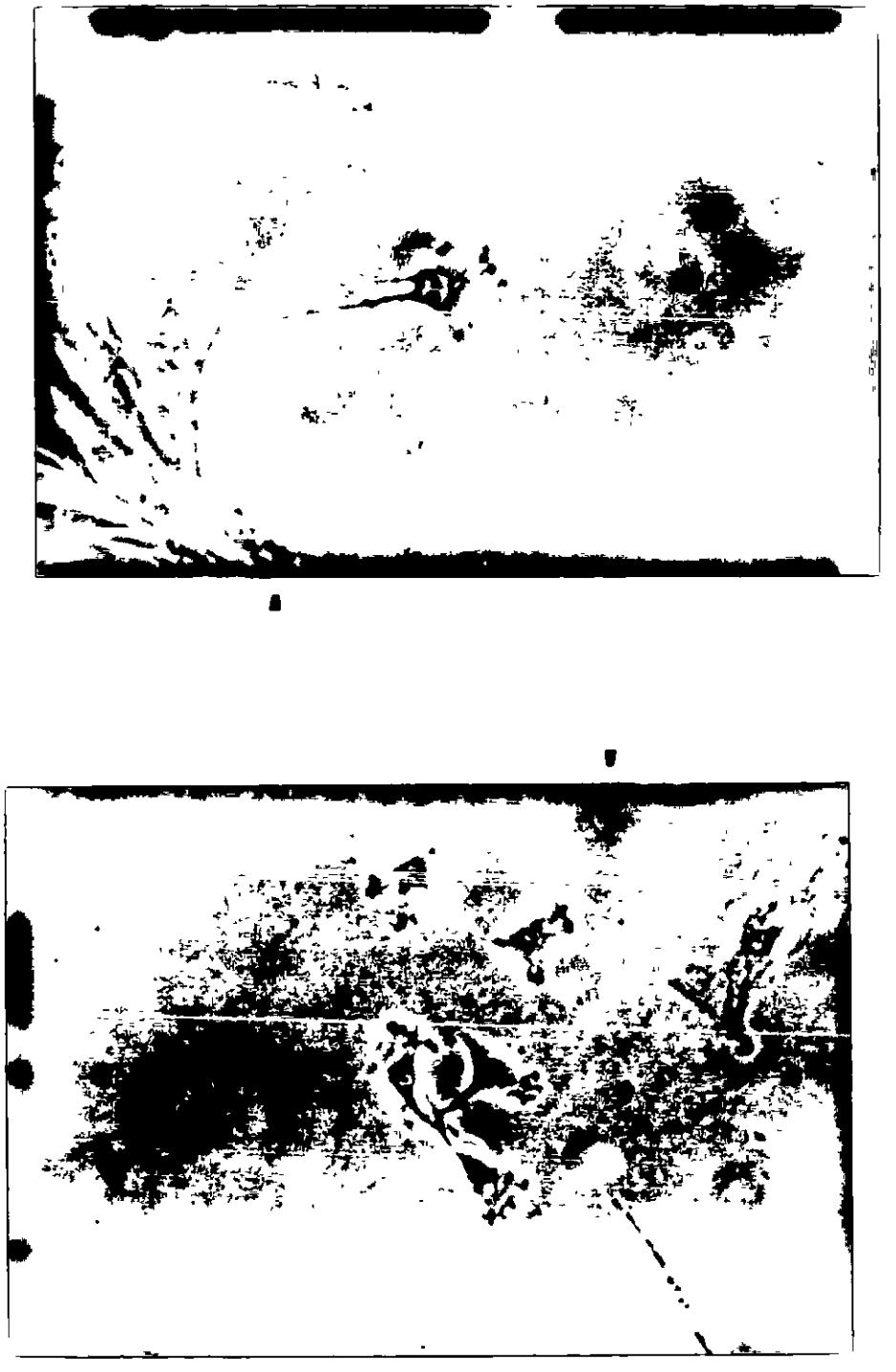


FIGURE 17

Photograph Of Mandibular Condyle Low Density



FIGURE 18

Photograph Of Septal Chondrocytes Low Density



FIGURE 19

Process Of Condylar Chondrocytes

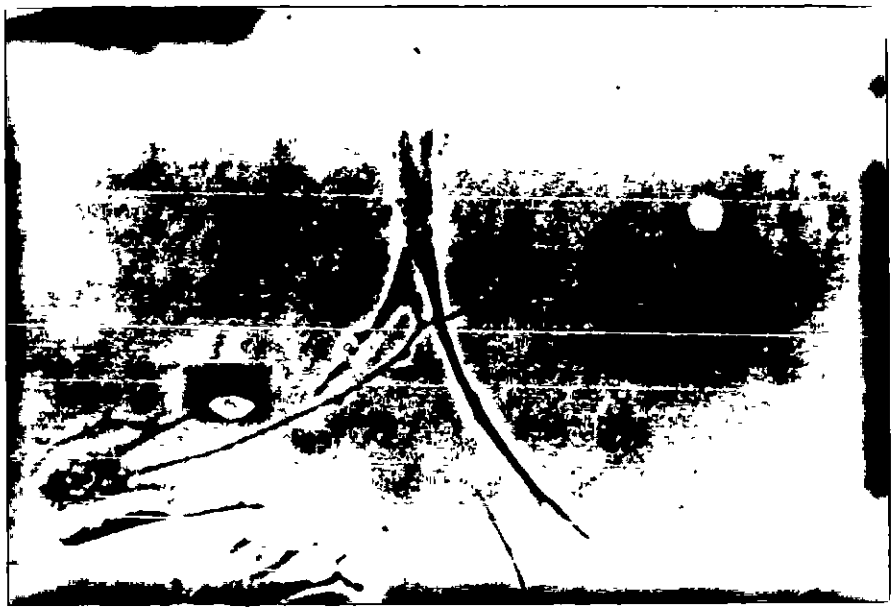


FIGURE 20

Photograph Of Nasal Septum Chondrocytes



FIGURE 21

Photograph Septal Process

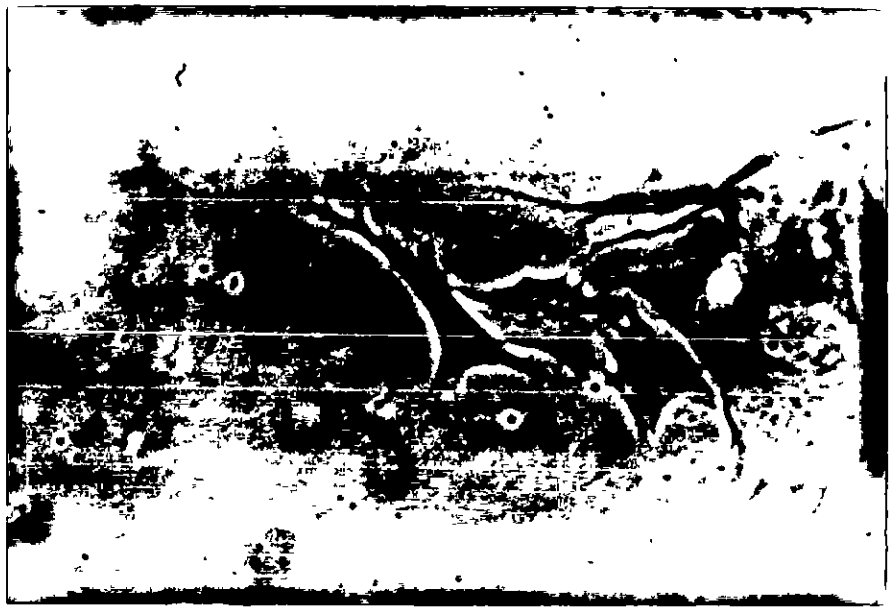
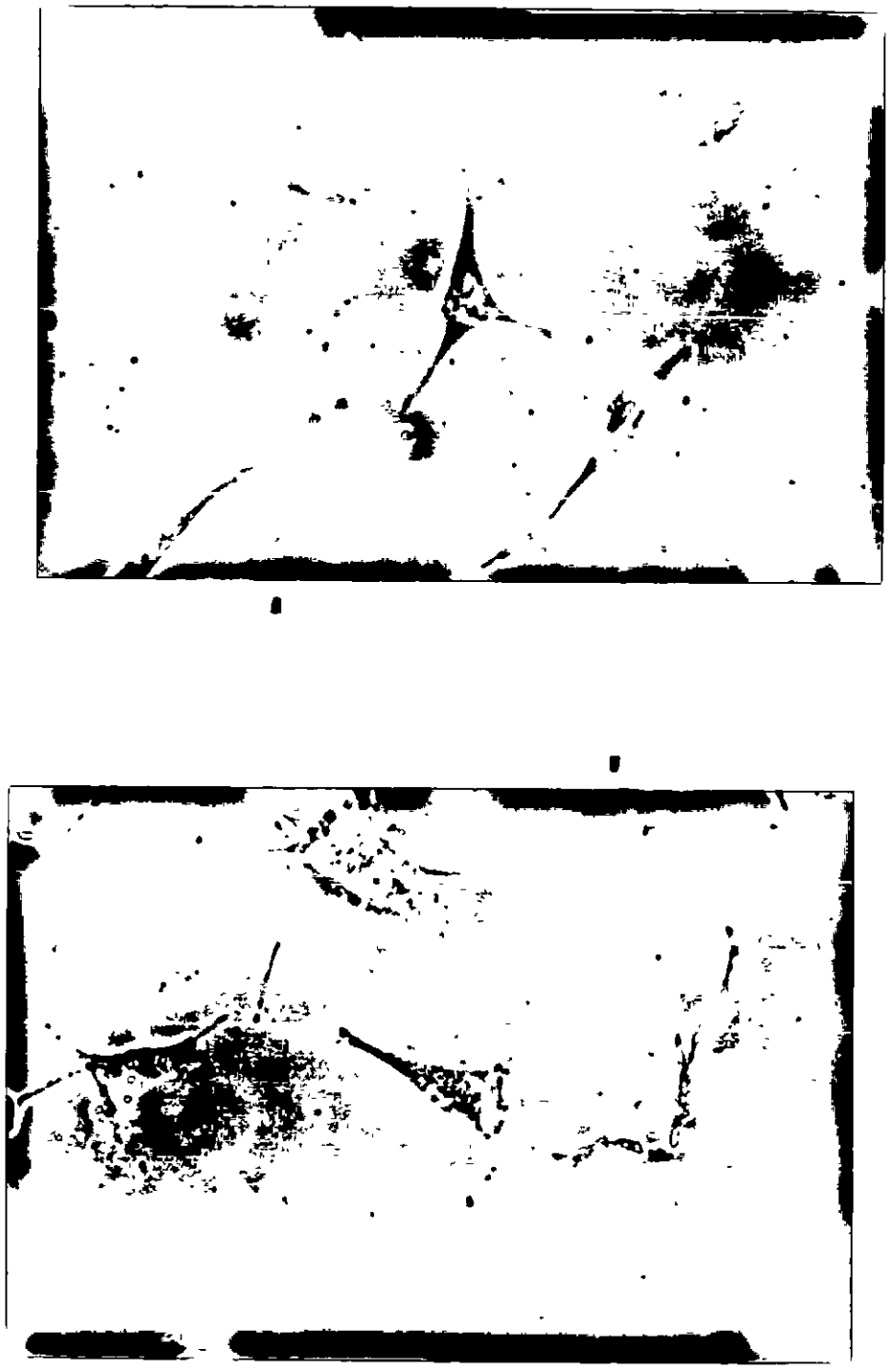


FIGURE 22

Photograph Of Epiphyseal Chondrocytes



DISCUSSION

The results of the comparison of cartilage components (matrix and cell membrane proteins) from the mandibular condyle, nasal septum and epiphyseal plates have demonstrated that there are differences among them. Protein profiles of the extracellular matrix of these cartilages demonstrate unique bands in each of the samples. The significance of this information is twofold: (1) It would be possible to produce monospecific antibodies, which could be used in vivo, to specifically study one cartilage without affecting others, and (2) The production of such a specific method of studying cartilage could ultimately lead us to have the ability to alter the growth of the mandibular condyle, the nasal septum, as well as other cartilages. This could aid orthodontists in the correction of skeletal discrepancies in both class II and class III malocclusions, without interfering with other tissues, the need for surgical correction of these conditions would be reduced.

Monospecific antisera raised against matrix components can be cytotoxic to chondrocytes. This has been demonstrated by T. Glant (1985) therefore specific tissues could be examined as to there role in the growth process. This technique could be used to examine the role of the nasal septum or the mandibular condyle in facial growth. Another important finding of this study is the instability of chondrocytes in culture. Under the various culture conditions used in this study, chondrocytes may have dedifferentiated very rapidly Specifically, membrane proteins isolated from fresh chondrocytes and allowed to recover for twelve and twenty

four hours, demonstrated important changes in several major high and low molecular weight protein bands. With increasing time of recovery it was observed that the prominence of these bands in the 100,000 dalton, 70,000 dalton, and 25,000 dalton range became either decreased or absent. An additional 122,000 dalton protein from the twenty four hour and cultured samples of the mandibular chondrocytes was also absent. These bands then remained decreased or were totally absent from cultured chondrocytes as well. It has been recognized by J. Mollenhaur (1982) and T. Glant (1985) that chondrocytes subjected to proteolytic enzymes during isolation and separation from whole cartilage require time to reconstitute there membrane proteins before they can be analyzed biochemically. It was apparent from the results of this study that there were obvious changes as little as twenty four hours after the time of isolation. To date there have been no standards set for the recovery periods. Many have recommended various time periods from one to twenty four hours. Without standardization it is axiomatic that the results of electrophoretic assays of protein profiles from different laboratories will be different. Several proteins present in the twelve hour samples were absent from twenty four hour and from cultured samples. These results indicate that proteins present in whole tissue may be absent from cells grown in cultures. Cell death may also explain the shift in molecular weight ranges seen between freshly isolated and cultured cells. Cell death and subsequent lysis releases intracellular proteases which may digest some of the available protein. This activity would lead to significant alterations in protein profiles such as that reported in the results of this study. Regardless of the cause of the protein shift, whole tissues should be used

for anticartilage antibody preparation. The use of whole tissue will ensure that the antibody is directed against a protein that is present in the growing animal.

Although the morphology of condylar, nasal septum and epiphyseal chondrocytes grown in culture is distinctly different, the protein profiles are surprisingly similar. This may be the result of the ratios of nutrients available in the culture media. Another possible explanation is a natural selection of more simplified cell forms best fit for survival in culture. In either case, caution should be used when comparing information obtained from studies done with cultured cells to information based on the study of cells obtained from whole tissue.

Extracellular matrix proteins were compared from freshly isolated chondrocytes and from cultured chondrocytes. Again it was found that matrix proteins produced by cultured cells differed significantly from those of whole tissue. In addition, obvious differences existed among matrix proteins produced by mandibular condylar, nasal septal, and epiphyseal chondrocytes. These differences were observed for both cultured and whole tissue. Differences were noted in several molecular weight ranges and all differences reported were unique to a single cartilage. Several bands appeared on two of the three and not the third and were therefore not considered to be unique. However, this too describes differences between the matrices of the three tissues examined. The absence of a protein from one cartilage matrix could also be exploited in the manipulation of that cartilage. The nature of the different proteins was not determined in this study. However, because of the isolation methods used for this study it can be concluded that they were not

proteoglycan fractions. Proteoglycans have very large molecular weight. Because our samples were not digested before electrophoresis, it can be assumed that the proteins that entered the gels were minor noncollagenous matrix proteins. Differences seen between whole and cultured matrix can be presumed to result from dedifferentiation of chondrocytes during the culturing process. Although Watt (1988), Handly (1978) and Benya (1982) have indicated that the dedifferentiation process can be prevented by using higher seeding densities and monolayer cultures, our results do not support these findings. Chondrocytes seeded at $1.3 \times 10^6/cm^2$ and grown in monolayer cultures were found to produce extracellular matrix. Differences caused by extended culture times were not examined.

Our results suggest that matrix proteins would be the most likely area from which to isolate unique proteins for the future production of monospecific antibodies. Glant (1988) demonstrated that antimatrix antibodies can be cytotoxic to chondrocytes and therefore, monospecific antibodies produced to specific matrix proteins could be used to alter cartilage growth.

Significant morphologic differences among the three populations of chondrocytes were also seen. Chondrocytes seeded at low density displayed highly variable morphology. This may mean that there are distinct subpopulations of chondrocytes within each tissue. Silbermann et al., (1973) had previously suggested that subpopulations of chondrocytes existed in the mouse mandibular condyle, and proposed that each subpopulation served a unique functional role. On the other hand diverse differences may be merely variations in cytoplasmic projections as

the chondrocytes attempt to achieve confluency. To make this determination chondrocytes would have to be subcultured individually. Individually cultured cells would then give rise to specific populations one could then determine if in fact they were distinct. However other problems would then arise, such as further dedifferentiation at extremely low seeding density. It becomes apparent that many variables such as type of culture media and seeding density, influence the results of cell and tissue studies. As mentioned previously several investigators were able to alter the constitution of matrix proteins by varying the concentration of certain elements in the media. Time in culture has also been shown to alter the constitution of matrix proteins as well. With all the important aspects to consider when studying cell and tissue in culture much can still be learned from these studies. If variables are controlled, and similar conditions are used, comparisons can still be made between individual tissues. However, care must be taken not to draw direct inferences to whole tissue or to living organisms containing those tissues, from in vitro cell culture studies alone. It must be demonstrated that the cultured cells are as biochemically similar as possible to whole tissue before such inferences can be drawn.

When comparing membrane proteins from chondrocytes of different anatomic origins, it was found that there were marked similarities among them. Virtually all major bands were identical among the cartilage chondrocytes examined in this study. Significantly, loss of major membrane protein bands with increasing time in the culture media occurred in chondrocytes from all three tissues examined. The same proteins were decreased or absent among all cell types. The exception to

this being condylar chondrocytes which lost an additional band in the higher molecular weight range, approximately 122,000 daltons. This suggests a difference in condylar chondrocytes compared to the nasal septum and epiphyseal chondrocytes. The significance of this difference is not clear at this time. However it can be speculated that the functional significance of this protein could be exploited in further studies involving condylar chondrocytes. The similarities in membrane protein profiles were surprising due to the striking morphological differences among them. It is possible that the methods of this study were not sensitive enough to detect significant differences among them. No attempt was made to examine the intracellular contents of the cells. It is possible that nuclear membrane proteins as well as intracellular proteins are present which would be unique to each cell population and therefore could also serve as antigens to produce monospecific antibodies. Another factor to consider in these findings is that the chondrocytes were considered as a unit from each tissue. Silberman et al., (1973) have demonstrated that there are unique subpopulations of chondrocytes present within each tissue. No attempts were made in this study to separate various subpopulations by either morphology or location within the tissue. Therefore, differences which may be present between them could not be detected.

Comparison of extracellular matrix proteins isolated from cultured chondrocytes demonstrated that there are major differences among the three cartilage types examined. In fact, each cartilage matrix sample demonstrated several unique proteins, not present in the other two. As mentioned previously, this study did not include proteoglycan fractions.

Samples were not digested prior to there being run on 7% SDS acrylamide gels. Without digestion of the samples, the large proteoglycan fragments will not enter 7% SDS acrylamide gels. The pour size of the 7% SDS acrylamide gels will allow for a maximum molecular weight of approximately 220,000 daltons. This is smaller then proteoglycan molecules in the undigested form. Proteoglycans have been demonstrated to have differences from one tissue to the next as well as age differences in the same tissue (Glant, 1985). It is likely that there may be additional differences among the three cartilages matrices due to differences in proteoglycan composition. Even without considering proteoglycan differences, it was still found that other minor matrix proteins varied from one tissue to the next. This indicates that cartilages from various anatomic locations are quite different. When comparing the nasal septal cartilage, a hyaline cartilage, to the mandibular condyle, a secondary cartilage, these differences might be expected. However, epiphyseal cartilage is also considered a primary hyaline cartilage and several major proteins were unique to the nasal septum and to the epiphyseal extracellular matrix. It would be interesting to examine other craniofacial cartilages, such as the sphenooccipital synchondrosis and the various sutures, to see how they compare to the nasal septum and the mandibular condyle.

The results of the microscopic examination of cultured chondrocytes indicated significant morphologic differences among the chondrocytes. When seeded at high density, chondrocytes harvested from each of the cartilages examined assume a morphology representative of the tissue of origin. That is to say that each cartilage was recognizable based on its

chondrocyte morphology. Chondrocytes of the mandibular condyle assumed a fibroblastic appearance and were organized into tightly packed sheets which extended in the direction of the cell's linear configuration. The chondrocytes of the mandibular condyle arrange themselves in an interlocking quarter to half staggered arrangement. Cell boundaries were not as clearly defined as in the nasal septum or epiphyseal cartilages. In contrast, the nasal septum chondrocytes assume a dense clustered configuration in which all cells appeared to assume a very similar size and shape. Cell boundaries were clearly visible no matter how dense the clusters became. Each nasal septum chondrocyte assumed a rounded to pentagonal shape and there relation to other cells was of equal distance on all sides. The chondrocytes of the nasal septum appeared to have a very granular cytoplasm with the nuclei displaced to one side.

The epiphyseal chondrocytes appeared in high density culture to be the least organized. The cells did not arrange themselves in any recognizable fashion. There appeared to be large irregular spaces between cells even at very high density. The most typical shape of epiphyseal chondrocytes was an oblong triangular appearance with the base of the triangle being the narrowest dimension of the cell. They appeared to possess a major cell process at the opposite pole from the base, which extended into the mass of cells. At the base of the cell each corner possessed a smaller process which did not extend significantly from the cell.

These differences in cell morphology suggest that cartilage is a heterogeneous tissue at both the biochemical and cellular level. It is hoped that these differences will be examined further because of the importance of this tissue to the facial growth process.

CONCLUSIONS

Several important implications can be drawn from this study. First, the extracellular matrix of cartilage derived from the mandibular condyle, nasal septum, and epiphyseal plates, each contain unique proteins. Because these proteins can act as antigens it would be possible to produce antibodies specific to each cartilage. The potential for creating a site specific anticartilage antibody offers unlimited possibilities for examining the facial growth process. For example, these antibodies could be used to examine the role of each craniofacial cartilage in the overall facial growth process. Each cartilage could be more specifically examined, eliminating many extraneous experimentally induced variables, that in the past have always been the subject of debate. Surgical extirpation could be replaced by specific cytotoxic antibodies that eliminate a specific cartilage from the growth process without altering adjacent tissues (muscle, bone neural and vascular). The roles of individual proteins in the growth process could also be examined. The identification of unique proteins utilized for these types of studies would necessarily lead to the identification of there specific roles. Antibodies with specific cartilaginous targets could further be conjugated with growth hormones or other growth promoting agents. This could allow for the treatment of skeletal malocclusions by altering the growth of specific bones. For example the mandibular condyle in skeletal class II cases. The prospects for clinical applications are exciting.

These results suggest that cartilage matrix proteins appear to be the most likely target for monospecific antibodies. Unique proteins were not

found on the membranes of chondrocytes. Chondrocytes of all three cartilages examined were distinctly different. They responded to experimental conditions differently, were morphologically different, and the matrix proteins produced were different. The results of this study also illustrate the importance of culture time and conditions, because the protein profiles of chondrocytes changed with increasing time in culture. Within twenty four hours after being isolated from whole tissue it was found that chondrocytes decreased or stopped the production of at least three major protein bands. This is important not only for the the information presented here, but in all studies employing cultured chondrocytes. There is no standard at the present time for the methods, amount of time in culture, or recovery period that is consistently utilized in the study of cartilage. Results obtained from different laboratories may not reflect true differences in results, as much as they may reflect differences in technique. This was also true of the matrix produced by cultured chondrocytes. When comparing the protein profiles of matrix produced in culture to that obtained from whole tissue, they were found to be very different. It was not determined at what time interval these changes could be observed or if further changes occurred with increasing time in culture. These observations must be taken to account for some of the differences that have been reported from various researchers. Again it should be noted that some standardization should be developed for the study of cultured tissues. Due to the changes that were observed between twelve and twenty four hour samples, the standard should be set at twelve hours or less.

Further conclusions can be drawn from the morphological aspects of this study. Chondrocytes displayed wide variations in morphology based on their tissue of origin. Morphology was also affected by seeding density. In high density cultures chondrocytes could be recognized by there tissue of origin, however, in low density cultures there were wide variations, not only between chondrocytes of different tissues, but with-in chondrocytes from the same tissue. This is of importance when examining information relating to cell morphology, because morphology can be altered simply by altering the number of cells seeded to primary cultures. There are numerous studies reporting on the morphology of chondrocytes at specific seeding densities, and if these studies are to be duplicated,the densities must be replicated as well. It can also be assumed that many differences related to chondrocyte morphology reported by different investigators may in fact be related to seeding densities rather than actual distinctions in morphology.

The results of this study will be valuable to other investigators pursuing this line of research. In particular, tissue culture and protein isolation standards to obtain repeatable results must be established. The most important objective of this study was to determine if in fact there exist recognizable differences in the protein profiles of various cartilages. We have proven that differences do exist. With the advances in protein biochemistry these differences will be valuable in furthering our understanding of the structure and function of cartilage, as well as our ability to alter and control facial growth.

APPENDIX I

PROCEDURE – SILVER STAINING

Mix 40 ml (a), 36 ml (b), .12 ml, 4 ml (h) are mixed at room temperature to cast two 16 x 20 cm slabs in Bio-rad Protein II casting block. Samples are run at 20 m for 1 hour and 40 minutes for remainder of run — 4 hours.

1. Fix in 50% methanol — 12% acetic acid for 30 minutes (wash glass with acetone)

2. Soak the gel twice for 15 minutes each time in 5% methanol — 7% acetic acid.

3. Fix in 10% unbuffered glutaraldehyde for 30 min.

4. Rinse in double distilled water for 10 minutes, repeat twice, soak in gel in double distilled water for a minimum of two hours. Best overnight or repeat 10 min. changes till glutaraldehyde odor is gone.

5. Soak the gel in 50 ml of ammonical silver solution for 10 - 15 minutes, shake on a rotary shaker at 75 RPM.

6. Transfer gel to double diluted water twice for 5 minutes each; rinse bottom of dish well to prevent sticking.

7. Soak gel in 0.005% citric acid and 0.019% formaldehyde until staining becomes apparent.

8. Transfer gel to a large volume of double distilled water to prevent further staining.

9. Store in % acetic acid solution. Resolution okay for a few days.

SOLUTIONS

Prepared in hood

1. 10% glutaraldehyde
   150 ml 30 ml of 50% kodak solution
   125 ml H_2O

2. Ammonical silver solution
   Add in this order
   100 ml
   1.4 fresh ammonium hydroxide
   21.0 ml .36% NaOH
   (.776g/4.0 double distilled water)
   4.0 ml 19.4% AgNO
   Shake when adding, should turn brown
   then clear 73.6 ml double distilled water

3. 0.005% citric acid + 0.019% formaldehyde
   100 ml
   1.0 ml 0.5% citric acid
   99 ml double distilled water
   50 micro Liters 37% formaldehyde

NOTES AND JUSTIFICATIONS

Minimize background by prefixing with methanol and acetic acid. The solution fixes everything on the gel.

50% MeOH – 12% HAC fixes proteins
5% MeOH – 7% HAC removes SDS

Glutaraldehyde — fixes proteins and matrix makes gel fragile, loses flexibility. The fixation of the gel and the washes determine the intensity of the background and therefore the resolution. Make sure to completely wash out the glutaraldehyde.

Addition of citric acid and formaldehyde (the procedure where proteins stain) stop reaction when all bands appear. Clearly reaction does not stop immediately, a diffusion process occurs so stop reaction before the intensity is at desired level.

Ammonium hydroxide solution must be fresh or sill will precipitate and solution will be brown instead of clear.

Silver stained gel can be stored in double distilled water for a few days without loss of resolution provided it has been washed extensively.

APPENDIX II

SDS GEL SYSTEM
Modified From Neville, et. al. J. BIOL CHEM 246 (20), 6328-6334 (1971)

Solutions: Store in refrigerator for no longer than two weeks

A) Separation Gel Acrylamide (Use I for 5% Gel)
11 gm Acrylamide
0.1 gm N, N'-Methylenebisacrylamide
Water to 50 ml. Filter!

B) Separation Gel Buffer
11.42 gm Tris
HCl to pH 9.2
Water to 50 ml. Filter!

C) Stacking Gel Acrylamide
4.34 gm Acrylamide
0.14 gm N, N'-Methylenebisacrylamide (B18)
Water to 50 ml. Filter!

D) Stacking Gel Buffer
0.728 gm Tris
H_2SO_4 to pH 6.1 (add carefully, especially around pH 6.7 because drops off rapidly after that)
Water to 50 ml. Filter!

E) Upper Reservoir Buffer
4.95 gm Boric Acid
1.0 gm SDS
Tris to pH 8.64 (dissolve approximately 55 gm Tris in 200 ml water and use to pH to solution)

Water to 1000 ml

F) Lower Reservoir Buffer
51 gm Tris
HCl to pH 9.2 (approximately 30 ml 1N HCl)
Water to 1000 ml

G) Protein Sample Solution
0.728 gm Tris
1.0 gm Sucrose
0.5 gm SDS
2.5 ml B-mercaptoethanol or (for 10 mM dithiotreatol .0772 gm)
Trace of Bromphenol blue
H_2SO_4 add slowly and carefully
Water to 50 ml

H) Catalyst
0.04 gm ammonium persulfate in 10 ml water
Must be fresh that day

I) Acrylamide Solution For 5% Gel
4.9 gm Acrylamide
0.1 gm N, N'-methylenebisacrylamide
Water to 50 ml. Filter!

PROCEDURE

1) Gel tubes should be acid washed and coated with photo flo 1:50 and allowed to dry. They are then placed in prep. rack with lower ends covered with parafilm.

2) To prepare lower gel, mix:
10.0 ml (A) + 9.0 ml (B) + 0.030 ml temed

then divide into 2 equal (9.5 ml) parts. To one of these add 0.5 ml (H), mix, and immediately fill six gel tubes to the mark, with the solution loosening any air bubbles caught at the bottom.

3) Layer gels carefully and quickly with distilled water (approximately 8 mm height).

4) Repeat (2), (3), for next six tubes.

5) After gels have polymerized (45 min.) add an equal volume of (B) to the water layer on gels and gently mix. let gels sit approximately two hours before pouring stacking gel.

6) Prepare stacking gel:
10.0 ml (C) + 9.0 ml (D) + 0.030 ml Temed
mix well, divide into two 4.5 ml volumes and one 10 ml volume.

7) Drain buffer from top of gels and rinse gel surface GENTLY with part of the 10 ml.
Drain well.

8) To one of the 4.5 ml volumes, add 0.5 ml (H), and mix.
Quickly add 0.2 ml (automatic pipetter) of this solution to each of the first six gel tubes and quickly layer with distilled water.
Stacking gel solution has a higher ratio of crosslinking and polymerizes quickly so speed is essential.

9) Repeat for the next six tubes.

10) Allow the gels to polymerize for one hour. Incubate protein samples one hour in 70° water bath.

11) Drain water form tubes and put into the electrophoresis apparatus. Do not mix the two sets of six tubes.

12) Layer protein solution on gels (max of 200 µl) and overlay with electrode buffer.

13) Electrophorese at 1/2 ma/tube for 1 hour then increase to 1 ma/tube until dye front nears the bottom of the tube.

14) When finished, remove tubes form apparatus remove gels from tubes using a stream of water through an 18 gage needle and place gel in the stain of a fixative.

(Solution x4 for slab gel)

APPENDIX III

MOLECULAR WEIGHT STANDARDS FOR ELECTROPHORESIS

SIGMA CHEMICALS	
Albumin	~66,000 Daltons
Beta-Lactoglobulin	~18,400 Subunit, 36,800
Native	
Lysozyme	~14,300 Daltons
Ovalbumin	~45,000 Daltons
Pepsin (Hog Stomach)	~34,700 Daltons
Trypsinogen	~24,000 Daltons
Bromphenol Blue	~669.98 Daltons
COMBUTEK	
RNA Polymerase	
Beta 1	~165,000 Daltons
Beta	~155,000 Daltons
Alpha	~39,000 Daltons
Albumin Rinder Serum	~68,000 Daltons
Trypsin Inhibitor	~25,000 Daltons

APPENDIX IV

OTHER EXPERIMENTAL FINDINGS

Chondrocytes of the mandibular condlye did very poorly in culture. The reason for this was unknown. Possible explanations were inadequate conditions such as the culture media and pH variables. During the culturing phase the pH will very between feeding intervals as growing cells consume oxygen and release by-products. It is possible that these small fluctuations in pH had significant effects. Chondrocytes of the mandibular condyle usually grew the fastest for the first few days in culture and then began to degenerate rapidly. The optimum time for collecting both cells and matrix was approximately 10 days. This lead to difficulty in collecting significant quantities of cells and extracellular matrix from each culturing cycle.

Four molar guanadine extraction of the extracellular matrix indicated experimentally that the extracellular matrix of the nasal septum was more highly hydrogen bonded. This was apparent due to the difficulty in detaching the cells from the culture flasks. Cells of the mandibular condyle detached almost immediately from the culture flasks during the harvesting step, followed by the epiphyseal and finally by the nasal septum.

Extraction of membrane proteins was another area of difficulty. At first ionic detergents and sonication were used with very little success. Membranes would not dissociate under these conditions, therefore no protein was available to enter the gels. A non-ionic detergent was then

employed to dissociate membranes with much greater success. The staining procedures were also modified during the coarse of this study. Preliminary staining procedures employed commassie blue, a protein stain frequently used to stain electrophoretic gels. It was observed that very little protein could be detected in the gels making comparison very difficult. Silver staining procedures were then used with much greater success. Silver stain is fifty to one hundred times more sensitive to protein when compared to commassie blue. Proteins which were previously not apparent became easily detectable making the results of this study possible to interpret.

Other differences were noted in matrix samples when being prepared for electrophoresis. In this preparation matrix samples were mixed with a protein sample solution. This sample solution contains bromphenol blue as a tracking die for electrophoresis. Bromphenol blue is also a pH indicator and changes from blue to yellow at pH 4.6. When preparing samples for the nasal septum and epiphyseal cartilages, the pH dropped from pH6.1 to change the color of the solution from blue to yellow. This was not true for the matrix samples for the mandibular condyle. The change was observed to be more rapid and higher in degree for septal matrix as opposed to epiphyseal matrix, indicating that septal matrix is more acidic then both epiphyseal and condylar matrix. These findings would further indicate that there important differences among extracellular matrix derived from cartilage of different anatomic locations.

The primary purpose of this study was to examine the cartilages of the mandibular condyle, nasal septum and epiphyseal plates for unique proteins which could be used for the production of monospecific antibodies. These monospecific antibodies could then be used to study the role of cartilage in the growth of the craniofacial complex. This study demonstrated that such proteins exist. In our laboratory we have anti sera raised in New Zealand white rabbits to the following cartilage components: (1) mandibular condyle chondrocytes, (2) condylar chondrocyte matrix, (3) nasal septal chondrocytes, (4) nasal septal chondrocyte matrix, (5) epiphyseal chondrocytes, and (6) epiphyseal chondrocyte matrix. At this time work is underway to screen these antisera for the presence of antibodies which may react with unique proteins of the matrix of the mandibular condyle. CsCl gradients of freshly isolated extracellular condylar matrix are being prepared for gross screening of the antisera. After adsorption to other cartilage tissues, the remaining antisera should contain antibodies specific to mandibular condylar cartilage. Separation of the various gradients and reaction to the antisera will elucidate molecules unique to the mandibular condyle. From this point, isolation and characterization of these proteins will be carried out. Once isolated they could be used for the production of specific antibodies and subsequent growth studies.

BIBLIOGRAPHY

Babula, W.J.; Smiley, G.R.; Dixon, A.D.; (1970) The Role of the Cartilaginous Nasal Septum in Midfacial Growth. Amer. J. Orthod Sept: 250-262.

Baylis, M.T.; (1988) Synthesis and Extracellular Assembly of Proteoglycans in Human Articular Cartilage. Agents and Actions. 23: 34-35.

Bergey, E.J.; Levine, M.J.; Reddy, M.S.; Bradway, S.D.; Al-Hashimi, I.; (1986) Use of the photoaffinity Cross-Linking Agent N-Hydro–xysuccinimicyl-4 Azidosalicylic Acid to Characterize Salivary Glycoproteins-Bacterial Interactions. Biochem. J. 234: 43-48.

Bollen, A.M.; (1986) Aggregation of Proteoglycans in Growth and Articular Cartilages. M.S. Thesis Dept. of Orthodontics, University of Michigan, Ann Arbor, MI.

Brigham, G.P.; (1976) An Investigation of Antigenic Differences Among Condylar, Epipheseal and Nasal Septal Cartilages. M.S. Thesis Dept. Of Orthodontics, Case Western Reserve University, Cleveland, OH.

Burkhardt, H.; Kasten, M.; Rauls, S.; (1988) A Low Molecular Weight Serine Proteinase Inhibitor From Human Articular Cartilage. Agents and Actions 23: 60-62. Mikecz, K.; Glant, T.T.; Buzas, E.; Poole, A.R.; (1988) Cartilage proteoglycans as Potential Autoantigens in Human and Experimental Animals. Agents and Actions 23: 63-66.

Caterson, B.; Christner, J.E.; Baker, J.R.; (1983) Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. Monoclonal antibodies to cartilage proteoglycan. J. Biol. Chem. 258: 8845-8854.

Cates, G.A.; Holland, P.C.; (1978) Biosynthesis of Plasma Membrane Proteins During Myogenesis of Skeletal Muscle in Vitro. Biochem. J. 174: 873-878.

Champion, B.R.; Sells, S.; Poole, A.R.; (1983) Immunity to Homologous Collagens and Cartilage Proteoglycans in Rabbits. Immunology 48: 605-616.

Corvol, M.T.; Dumontier, M.F.; Garabedian, M.; Rappaport, R.; (1978) Vitamin D and Cartilage II Biological Activity of 25 Hydro—xycholecalciferol 24, 2 and 1, 25 Dihydroxycholecalciferols on Cultured Growth Plate Chondrocytes. Endocrinology 102 #4: 1269-1274.

Doege, K.; Sasuki, M.; Horigan, E.; Hassell, J.R.; Yamada, Yoshihiko; (1987) Complete Primary Structure of the Rat Cartilage Proteoglycan Core Protein Deduced From CDNA Clones. J. Biol. Chem. 262: 17757-17767.

Doege, K.; Fernandez, P.; Hassell, J.R.; Sasaki, M.; Yamada, Y.; (1986) Partial CDNA sequence encoding a Globular Doman at the C Terminus of the Rat Cartilage Proteoglycan. J. Biol. Chem. 261: 8101-8111.

Faissner, A.; Kruse, J.; Chiqyet-Ehrismann, R.; Mochie, E.; (1988) The High Molecular Weight J1 Glycoproteins are Immunochemically Related to Tenascin. Differentiation 37: 104-114.

Franzen, A.; Heinegard, D.; (1985) Isolation and Characterization of Two Sialoproteins Present Only in Bone Calcified Matrix. Biochem. J. 232: 715-724.

Fuhlendorff, J.; Clemmensen, I.; Magnusson, S.; (1987) Primary Structure of Tetranectin, Aplasminogen Kringle 4 Binding Plasma Protein Homology with Asialogylcoprotein Receptors and Cartilage Proteoglycan Core Protein. Biochemistry 26: 6757-6764.

Gertzbein, S.D.; Tait, J.H.; Devlin, S.R.; Argue, S.; (1977) The Antigenicity of Chondrocytes. Immunology 33: 141-145.

Gianelly, A.A.; Moores, C.F.A.; (1965) Condyectomy in the Rat. Arch Oral Biol. 10: 101-106.

Glant, T.T.; Mikecz, K.; Poole, A.R.; (1986) Monoclonal Antibodies to Different Protein-related Epitopes of Human Articular Cartilage Proteoglycans. Biochem. J. 234: 31-41.

Glant, T.T.; Mikecz, K.; Roughey, P.J.; Buzas, E.; Poole, A.R.; (1986) Age-related Changes in Protein Related Epitopes of Human Articular Cartilage Proteoglycans. Biochem J. 236: 71-75.

Glant, T.; Levai, G.; Hadhuzy, C.; (1977) The Localization of Proteoglycans and Glycoproteins in the Hyaline Cartilage. Histochemistry 53: 291-299.

Glant, T.; Levai, G.; (1983) Localization of Antigenic Components in Proteoglycan Aggregate of Bovine Nasal Cartilage. Histochemistry 77: 217-232.

Glant, T.; Mikecz, K.; (1986) Antigenic Profiles of Human, Bovine, and Canine Articular Chondrocytes. Cell Tissue Res. 244: 359-369.

Glant, T.; Mikecz, K.; Leva, G.; (1981) The Cell Surface of Antigens of Articular Chondrocytes. Semin. Arthritis Rheum. (Supplemental) II: 113-114.

Golds, E.E.; Stephen, I.B.M.; Esdaile, J.M.; Stranczynski, H.; Poole, A.R.; (1983) Lymphocyte Transformation to Connective Tissue. Antigens in Adult and Juvenile Rheumatoid Arthritis, Osteoarthritis, Spondylitis Systemic Lupus Erthemotosos and a Non-arthritic Control Population. Cellular Immunology. 82: 196-209.

Handley, C.J.; Lowther, D.A.; (1979) Extracellular Matrix Metabolism by Chondrocytes 5. The Proteoglycans and Glycosaminoglycans Synthesized by Chondrocytes in High Density Cultures. Biochim Biophy Acta. 582: 234-245.

Hans, M.G.; (1981) The Effects of Antinasal Septal Cartilage Antibodies on Midfacial Growth and Development. M.S. Thesis Dept. Of Orthodontics, Case Western Reserve University, Cleveland, OH.

Harper, G.S.; Hascall, V.C.; Yanagishita, M.; Gahl, W.A,; (1987) Proteoglycan Synthesis in Normal and Howe Syndrome Fibroblasts. J. Biol. Chem. 262: 5637-5643.

Hascall, V.C.; Riolo, R.L.; Hayward, J.; Reynolds, C.C.; (1972) Treatment of Bovine Nasal Cartilage Proteoglycan With Condroitinase from Flavobacterium Heparinum and Proteus Volgaris. J. Biol. Chem. 247: 4521-4528.

Hascall, G.K.; (1979) Cartilage Proteoglycan Comparison of Sectioned and Spread Whole Molecules. J. Ultrastructure Research. 70: 369-375.

Hascall, G.; Nishimura, I.; Konomi, H.; Vander Rest, M.; Ninomya, Y. (1988) Cartilage Type 1X Collagen Proteoglycan Contains a Large

Amino Terminal Globular Doman Encoded by Multiple Exons. J. Biol. Chem. 263: 2324-2329.

Heinegard, D.; Paulson, M.; Inerot, S.; Carlstrom, C.; (1981) A Novel Low Molecular Weight Chondroitin Sulphate Protoeoglycan Isolated From Cartilage. Biochem. J. 197: 355-366.

Heinegard, D.; Hascall, V.C.; (1974) Aggregation of Cartilage Proteoglycan III Characteristics of the Proteins Isolated From Trypsin Digests of Aggregates. J. Biol. Chem. 249: 4250-425

Yip, D.K.; Averspery, N.; (1972) The Dye-Exclusion Test for Cell Viability: Persistence of Differential Staining Following Fixation. In Vitro 7: 323-329.

Hans, M.G.; (1981) The effects of Antinasal Septal Cartilage Antibodies on Midfacial Growth and Development. M.S. Thesis Dept. Of Orthodontics, Case Western Reserve University, Cleveland, OH.

Hewitt, T.A.; Varner, H.H.; Silver, M.H.; Dessau, W.; Wilkes, C.M.; Martin, G.R.; (1982) The Isolation of Partial Characterization of Chondronectin, an Attachment Factor for Chondrocytes. J. Biol. Chem. 257: 2330-2334.

Hjertquist, S.O.; Wasteson, A.; (1972) The Molecular Weight of Chondroitin Sulphate From Human Articular Cartilage. Calcif. Tissue Res. 10: 31-37.

Hunter, G. K,; Rogakov, C.C.; Pritzker, K.P.; (1984) Extracellular Matrix Synthesis by Articular Chondrocytes and Synovial Fibroblasts in Long Term Monolayer Culture. Biochim Biophy Act. 804: 459-465.

Kantomaa, T.; (1987) Effect of Functional Change on Cell Differentiation in The Condylar Cartilage. J. Anat. 152: 133-143.

Klasen, I.S.; Ladestein, R.M.T.; Van den Borg, W.B.; Benner, R. (1988) Flare up of Joint Inflammations Induced by Cloned Helper T-Cells. Retention of the Helper-T Cells. Agents and Action 23: 69-73.

Kleinman, H.K.; Klebe, R.S.; Martin, G. R. (19) Role of Collagenous Matrices in the Adhesion and Growth of Cells. J. Cell Biol. 88: 473-475.

Koski, K.; Odont, L; Odont, D.; (1968) Cranial Growth Centers —Facts or Fallacies? Am. J. Orthod Aug: 566-583.

Kotyla, J.R.; (1986) An Exploratory Study of The Mandibular Condyle in the Rat Following Changes in Articular Function. Eur. J. Orthod 8: 265-270.

Landsberg, C.R.; Yavin, E.; (1979) Protein Profiles of Rat Embryo Cerebral Cells During Differentiation in Culture. J. Neuro. Chem. 32: 133-143.

Landsberg, C.R.; (1988) Nerve Growth Factor-Induceable, Large External (NILI) Glycoprotein in Developing Rat Cerebral Cells in Culture. Cell Tissue Res 252: 181-190.

Meikle, M.C.; (1973) In Viro transplantation of the Mandibular Joint of the Rat; An Autoradiographic Investigation Into Cellular Changes at the Condyle. Arch. Oral. Biol. 18:1011-1020.

Mollenhauer, J.; Bee, J.A.; Lizarbe, A.; Vander Mark, K.; (1984) Role of Anchorin CII a 31,000 mol-wt Membrane Protein in the Interaction of Chondrocytes With Type II Collagen. J. Cell Biol. 98: 1572-1578.

Mollenhauer, J.; Brine, K.; (1988) Detection of Autoimmuno reactive Antibodies Against Cartilage Cell Surface Proteins in the Blood of Rheumatoid Patients. Agents and Actions. 23: 48-49.

Morales, T.I.; Hascall, V.C.; (1988) Correlated Metabolism of Proteoglycans and Hyaluronic Acid in Bovine Cartilage Organ Cultures. J. Biol. Chem. 263: 3632-3638.

Moss, M.L.; Bromberg, B.E.; Song, I.C.; Eisenman, G.; (1968) The Passive Role of Nasal Septal Cartilage in Midfacial Growth. Plastic and Reconstructive.

Moss, M.L.; Salentign, L.; (1971) The Compensitory Role of the Condylar Cartilage in Mandibular Growth: Theoretical and Clinic Implications. Dtsch Z Mund Kiefer Gesichtschir. 56: 5-16.

Nakazawa, K.; Suzuki, N.; Suzuki, S.U.; (1975) Sequential Degredation of Keratan Sulfate by Bacterial Enzymes and Purification of a Sulfabase in Enzymetic System. J. Biol. Chem. 250: 905-911.

Nakazowa, K.; Suzuki, S.; (1975) Purification of Keratan Sulfate-Endogalactosidase and its Action on Keratan Sulfates of Different Origin. J. Biol. Chem. 250: 912-917.

Neane, P.J.; Christner, J.E.; and Baker, J.R.; (1987) Cartilage Proteoglycan Aggregates The Link Protein and Proteoglycan Amino-term: Globular Domains Have Similar Structures. J. Biol. Chem. 262: 17768-17778.

Palmer, N.S.; (1978) Invetro and Invivo Study of Anticartilage Antibodies. M.S. Thesis Dept. Of Orthodontics, Case Western Reserve University, Cleveland, OH.

Paulsson, M.; Heinegard, D.; (1981) Purification and Structural Characterization of a Cartilage Matrix Protein. J. Bio. Chem. 197: 367-375.

Perih, J.A.; (1989) Limited Characterization of Rat Cephalic Cartilages. M.S. Thesis Dept. Of Oral Biology, Case Western Reserve University, Cleveland, OH.

Poole, A.R.; Reiner, A.; Tang, L.H.; Rosenburg, L.; (1980) Proteoglycans From Bovine Nasal Cartilage Immunochemical Studies of Link Protein. J. Biol. Chem. 255: 9295-9305.

Poole, A.R.; Pidoux, I.; Reiner, A.; Rosenburg, L.; (198) An Immunoelectron Microscope Study of the Organization of Proteoglycan Monomer, Link Protein, and Collagen in the Matrix of Articular Cartilages. J. Cell Biol. 93: 921-937.

Prehm, P.; (1988) Biosynthesis of Hyaluronate. Agents and Actions. 23: 36-37.

Ratcliffe, A.; Hardingham, T.; (1983) Cartilage Proteoglycan Binding Region and Link Protein Radioimmunoassays and the Detection of Masked Determinants in Aggregates. Biochem. J. 213: 371-3.

Renart, J.; Reiser, J.; Stark, G.R.; (1979) Transfer of Proteins From Gels to Diazobenzyloxymethyl Paper and Detection With Antisera: A Method of Studying Antibody Specifically and Antigen Structure. Proc. Natl. Acad. Sci. 76: 3116-3120.

Ruggiero, F.; Pfaffle, M.; Vander Mark, K.; Garrsone, R.; (1988) Retention of Carboxypropeptides in Type II Collagen Fibrils in Chick Embryo Chondrocyte Cultures. Cell and Tissue Res. 619-624.

Salentign, L.M.; Moss, M.L.; Shinozuka, M.; Skalak, R.; (1987) Morphological Analysis and Computer-aided Three Dimensional

Reconstruction of Chondrocytic Columns in Rabbit Growth Plates. J. Anat. 151: 157-167.

Schalkwijk, T.; Jooster, L.A.B.; Vanderburg, W.B.; Van de Putte, L.B.A.; (1988) Elastase Secreted by Activated Polymorpho-nuclear Leukocytes Causes Chondrocyte Damage and Matrix Degradation in Intact Acticular Cartilage. Agents and Actions 23: 58-59.

Sibermann, M.; Reddi, A.H.; Hand, A.R.; Leapman, R.; Vander-Mark, K.; Franzen, A. (1987). Chondroid Bone Arises From Mesenchymal Stem Cells in Organ Culture of Mandibular Condyles. J. Craniofacial Genetics and Development. Biol. 7: 59-79.

Silberman, M.; Reddi, A.H.; Hand, A.R.; Leapman, R.D.; VonDer Mark, K.; Franzen, A.; (1987) Further Characterization of the Extracellular Matrix in the Mandibular Condyle in Neonatal Mice. J. Anat. 151: 169-181.

Silbermann, M.; VonderMark, K.; Miroky, N.; Van Menxel, M.; Lewinson, D.; (1987) Effects of Increased Doses of 1.25 Dihydroxy Vitam D on Matris and DNA Synthesis in Condylar Cartilage of Suckling Mice. Calc. Tissue Inter. 41: 95-104.

Silbermann, M.; Frommer, J.; (1973) Heterogencity Among Chondrocytes of the Mandibular Condyle in Foetal and Postnatal Mice. Arch Oral Biol. 18: 1549-1554.

Sorrell, J.M.; Mahmoodian, F.; Caterson, B.; (1988) Immuno-chemical Characterization and Ultra Structure Localization of Chondroitin Sulfates and Keratan Sulfate in Embryonic Chick Bone Marrow. Cell Tissue Res. 252: 523-531.

Swann, D.A.; Powell, S.; Sotman, S.; (1979) The Heterogencity of Cartilage Proteoglycans. J. Biol. Chem. 254: 945-954.

Takigawa, M.; Okada, M.; Takano, T.; OHMAEH; Sakuda, M.; Suzuki, F.; (1984) Studies on Chondrocytes From Mandibular Condylar Cartilage. Nasal Septal Cartilage, and Speno-occipital Synchondrosis in Culture. I. Morphology, Growth, Glycosamino-glycan Parathyroid Hormone. (1-34) J. Dent Res. January, 1984: 19-22.

Thower, E.J.M.; Buckwalter, J.A.; Kuettner, K.E.; (1986) Maturation-related Differences in the Structure and Composition of

Proteoglycans Synthesis by Chondrocytes From Bovine Articular Cartilage.

Vanbeuningen, H.M.; Vandenburg, W.B.; Schalkwijk, J.; Arntz, O.J.; Vandeputte, L.B.A.; (1988) Age Related Changes in Joint Damage Caused by Antigen-Induced Arthritis in Mice. Agents and Actions. 23: 74-76.

Vankampert, G.P.J.; Van de Stadt, R.J.; Kiljan, E.; Brand, H.S.; Kuijer, R.; Van der Krost, J.K.; (1988) Effects of Tissue Dis-organization on Proteoglycans Synthesis by Articular Chondrocytes in Vitro. Agents and Actions 23: 40-41.

Vaughan, L.; Huber, S.; Chicquet, M.; Winkerhalter, K.; (1987) A Major Six-Armed Glycoprotein From Embryonic Cartilage. EMBO J. 6: 349-353.

Vertel, B.M.; Dorfman, A.; (1979) Simultaneous Localization of Type II Collagen and Core Protein of Chondroitin Sulfate Proteoglycan in Individual Chondrocytes. Cell Biology 76: 1261-1264.

Vertel, B.M.; Dorfman, A.; (1978) An Immunohistochemical Study of Extracellular Matrix Formation During Chondrogenesis. Developmental Biol. 62: 1-12.

Vonder Mark, K.; Mollenhauer, J.; Muller, P.K.; Pfaffle, M.; (1985) Anchorin CII, a Type II Collagen-binding Glycoprotein From Chondrocyte Membranes. Ann NY Acad. Sci. 460: 214-223.

Watt, F.M.; (1988) Effect of Seeding Density on Stability of the Differentiated Phenotype of Pig Articular Chondrocytes in Culture. J. Cell Sci. 89: 373-378.

Weiss, A.; Liune, E.; Brandeis, E.; Sibermann, M.; (1988) Triamcinolone Impairs the Synthesis of Collagen and Non-Collagen Proteins in Condylar Cartilage of Newborn Mice. Calc. Tissue Inter. 42: 63-69.

Werner, H.; Paegelow, I.; Vietinghoff, G.; (1988) The Production of Interleukin-1 Induced by an Acute Non-specific Inflammation in mice. Agents and Actions. 23: 79-81.

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