Morphologic, Microscopic, and Immunohistochemical Investigations Into the Function of the Primate TMJ Disc

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The distribution and arrangement of extracellular matrix proteins were examined in the primate temporomandibular joint disc and posterior attachment using a combination of light microscopic, immunohistochemical, and biochemical techniques. The band areas of the disc contain a complex collagenous (type I) fiber network consisting of a mediolaterally directed fiber bundle system that interlaces or becomes continuous with an anteroposteriorly directed collagenous fiber array that runs through the intermediate zone. Thin, branching, elastic fibers are a significant component of the disc and are generally oriented parallel to the collagenous fiber network. Interfibrillar spaces in band areas contain numerous chondrocytes encased within a matrix that is rich in a high molecular weight, predominantly chondroitin-sulfate proteoglycan and type II collagen. The intermediate zone appears tendinous in its construction and is composed of anteroposteriorly oriented elastic and collagenous fibers, scattered chondrocytes, and reduced amounts of chondroitin-sulfate proteoglycan and type II collagen. The posterior attachment is composed of fibrocytes, larger caliber elastic fibers, loosely organized type I collagenous fibers, and low molecular weight dermatan-sulfate proteoglycan. These results indicate that the primate temporomandibular joint disc is a microheterogenous tissue with distinct regional specializations.
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The physiochemical composition and geometric arrangement of extracellular matrix (ECM) proteins within a tissue determines its properties.[1–3] Tendon, for example, is primarily subjected to tensional stresses and is typically composed of small amounts of a low molecular weight dermatan-sulfate proteoglycan (DSPG) and parallel bundles of tightly packed, type I collagenous fibers oriented in line with the stress.[4–7] Hyaline articular cartilage, which absorbs and distributes compressive loads, is characterized by an abundant matrix rich in water and a high molecular weight chondroitin-sulfate proteoglycan (CSPG) constrained by a complex, type II collagenous meshwork.[8,9] Knowledge of the type and distribution of proteoglycan and of the fibrillar composition and arrangement in the ECM can therefore tell much about the forces acting on a tissue and assist in the formulation of a model for its mechanical function.[3,10–12]

The ECM of the temporomandibular joint (TMJ) disc is also a composite of proteoglycans and collagenous fibers and also contains notable numbers of elastic fibers. Accordingly, the disc should be expected to have material properties that are a function of the interaction of these elements. Similarly, regional differences in the quan-

tity and organization of discal constituents should be correlated with regional differences in its mechanical function.

While the shape and gross internal architecture of the TMJ disc have been the subject of numerous investigations,[13–22] relatively little work has been done in identifying the types and micro-organization of discal ECM proteins[17,23–31] and on their contribution to discal properties.[32–34]

The present study was undertaken to increase understanding of the organization of the human jaw joint disc. Use of human tissue is subject to many limitations, and studies on an animal having a disc with human features are essential if appropriate observations are to be made. Preliminary observations on the TMJ disc and posterior attachment (PA) in macaques and baboons established that these tissues are quite similar in form and composition to those of humans. The specific aims of the present investigation were to determine the distribution of proteoglycans and the arrangement of collagenous and elastic fibers in three areas of the primate disc—the anterior band (AB), posterior band (PB), and intermediate zone (IZ)—and in the PA. Light and polarizing microscopy were used to determine the arrangement of elastic and collagenous fibers, radiolabeling with [^{35}S]-sulfate was used to identify major proteoglycan types, and immunohistochemistry was used to assess the relative distribution of proteoglycans and types I and II collagen.

Materials and Methods

Fixation and Processing

Twenty-two juvenile (canines still erupting) and subadult (canines in occlusion) macaques *(Macaca fasciculata)* and baboons *(Papio cynocephalus)* were sacrificed at the Biological Resource Laboratory at the University of Illinois at Chicago. The jaw joints and one knee joint from each of these animals form the basis of this study (Table 1). A total of 12 primates (8 macaques, 4 baboons) were used for organ and monolayer culture and 10 (7 macaques, 3 baboons) were used for light and immunofluorescent microscopy. The joint was opened and the disc and attachment tissues were identified (Fig 1). The TMJ disc and PA were removed from both joints of each specimen within 30 minutes of sacrifice and placed in balanced salt solution (BSS) (Gibco, Grand Island, NY) containing a 5× concentration of antibiotic-antimycotic (Gibco). The specimens were viewed under a dissecting microscope and nonrelevant, adherent peridiscal soft tissue was removed. The specimens were then prepared for either light microscopy or organ and monolayer culture.

Table 1 Distribution of Animals

Group	N	Juvenile	Subadult	Male	Female
Macaca fasiculata	15	10	5	6	9
Papio cynocephalus	7	4	3	4	3
Total	22	14	8	10	12

Light Microscopy

The disc and PA were fixed overnight in 3% formalin. After fixation and overnight wash, each disc was subdivided and sectioned in a standard way, in parasagittal and horizontal planes (Fig 1). Each tissue block was dehydrated in graded ethanols, embedded in paraffin with anatomic orientations preserved, and serially sectioned at 6 μm. Observations on matrix content and organization were made in the central, lateral, and medial thirds within each region. For tissue identification, every fifth slide was stained with hematoxylin and eosin. The presence and distribution of glycosaminoglycan (GAGs) were identified using toluidine blue in acetate buffer at pH 4.2. The distribution and arrangement of mature elastic fibers were documented by staining with a modified Verhoeff's elastic stain.[29] Samples of monkey ear cartilage and associated tissues, used as controls for immunohistochemistry, were fixed and prepared for microscopy as described above. Photomicrographs for light microscopy were taken with Fujicolor 400 print film and for immunofluorescence microscopy with Kodak Tri-X 400 print film.

Collagen Fiber Analysis

Collagen is a birefringent tissue. The predominant orientation of the collagenous fiber bundles was determined by examination of unstained and toluidine blue–stained sections under circular polarizing light (CPL).

Antibodies

The immunofluorescence staining properties of the antibodies used in this study and other characteristics of their immunoreactivities have been previously described and characterized.[35–44] These antibodies included:

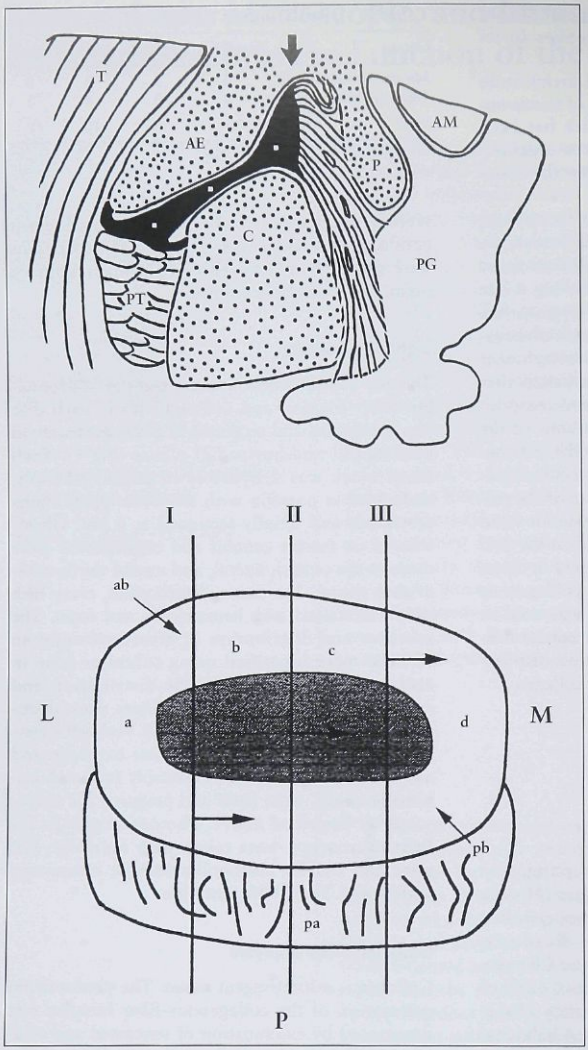

Fig 1 *(A)* Schematic drawing of parasagittal section through the TMJ of a subadult specimen of *Macaca fasciculata*. Anterior is to the left. The upper stippled region represents the articular eminence anteriorly and the postglenoid process posteriorly; lower stippled region is the mandibular condyle. The black region represents the disc and its anterior temporal and condylar attachments. Posterior expansion of the disc is the posterior band. The anterior band lies in front of the condyle and is continuous with the temporal and condylar attachments. The IZ of the disc lies between the condyle and eminence. The PB of the disc is continuous with the vascular posterior attachment, which is attached to the anterior surface of the postglenoid process, parotid gland, and posterior surface of the condyle. (AE = articular eminence; AM = auditory meatus; C = condyle; P = postglenoid process; PG = parotid gland; PT = lateral pterygoid insertion; T = temporalis muscle.) Black arrow marks posterior limit of the posterior band and its junction with the posterior attachment. Tissue samples for cell and organ culture of the disc were taken anterior to this point. White squares indicate sample areas in the disc. PA samples were taken well behind the PB. *(B)* Diagram of superior surface of disc and PA. Lines I, II, and III mark the planes of parasagittal sectioning for light microscopy. The arrows indicate the direction of sectioning of tissue blocks b, c, and d. Horizontal sections were taken parallel to the superior surface of the disc. (ab = anterior band; pb = posterior band; pa = posterior attachment; L = lateral; M = medial; P = posterior; shaded area = IZ.)

1. Monoclonal antibodies for link protein (designated 8-A4) and for the GAGs: chondroitin-six sulfate (designated 3-B-3), keratan-sulfate (designated 5-D-4), chondroitin-four sulfate (designated 9-A-2), and hyaluronic acid (designated 1-B-5)[35,37]
2. The monoclonal antibody for chondroitin sulfate (CS56, Sigma Chemicals), which is specific for the glycosaminoglycan portion of native chondroitin sulfate proteoglycan[36]
3. The monoclonal antibodies for type II collagen (CIICI),[38] which recognizes an epitope on the fibrous portion of the molecule, and for type I procollagen (SP1)[44] (Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa, Iowa City)
4. Polyclonal rabbit and guinea pig antibodies directed against chicken hyaluronidase-digested aggrecan monomer (RaCP, GpCP) and types I (GpIC) and II (GpIIC) collagen[39-42]
5. The polyclonal antibody for the cellular form of fibronectin (SFN, Sigma Chemicals)

The antibodies' specificity, characteristics, and working conditions are shown in Table 2.

Table 2 Specificity, Characteristics, and Conditions for Use of Various Antibodies

Antibody	Enzyme treatment	Antigen recognized
Glycosaminoglycans		
3-B-3	Chondroitinase ABC	Chondroitin-six sulfate
CS56	None	Chondroitin-six sulfate
9-A-2	Chondroitinase ABC	Chondroitin-four sulfate
5-D-4	None	Keratan sulfate
Chondroitin-sulfate proteoglycan		
GpCP	None	CSPG core protein
RaCP	None	CSPG core protein
Types I and II collagen		
GpIC	Trypsin/Hyaluronidase	Type I collagen
SP1	Trypsin/Hyaluronidase	Type I collagen
GpIIC	None	Type II collagen
CIICI	Trypsin/Hyaluronidase	Type II collagen
Cartilage matrix products		
1-B-5	Hyaluronidase	Hyaluronic acid
8-A-4	None	Link protein
SFN	None	Fibronectin

Immunofluorescence Microscopy

The composition of the disc's matrix was further characterized by indirect immunofluorescence. Tissue samples were processed, embedded, and sectioned as outlined above. Indirect immunofluorescent localization was performed on tissue sections using the following procedures and reagents.

Sections were deparaffinized and brought to buffer. In cases where antibody application was preceded by enzymatic digestions, sections were incubated with various enzymes (Table 2). *Streptomyces* hyaluronidase was used at 200 U/mL in 0.02 M sodium acetate (pH 5.2) and 0.01% bovine serum albumen (BSA), and it was applied to sections for 2 hours at 37°C. Chondroitinase ABC and ACII were used at 0.1 U/mL in 0.1 M sodium acetate and 0.1 M Tris-HCl (pH 7.3), and it was applied to sections for 2 hours at 37°C. Trypsin was used at 0.1% in 0.01 M phosphate buffer (PBS)/0.1% calcium chloride and applied to sections for 10 minutes at room temperature. Sections were then quick washed in PBS (pH 7.4) containing 0.1% BSA. Five percent normal goat serum in PBS was added to each section for 30 minutes at room temperature. Primary antibodies (diluted 1:25 to 1:150 in PBS, pH 7.4, containing 0.1% BSA) were applied to sections and allowed to incubate overnight at 4°C. Sections were washed six times over 30 minutes in PBS (pH 7.4) containing 0.1% BSA.

Antigen-antibody binding was visualized by indirect immunofluorescence using Ig-class and species-specific secondary antibodies directly conjugated to fluorescein or rhodamine (diluted 1:25 to 1:100 in PBS pH 7.4 containing 0.1% BSA). The sections were washed six times over 30 minutes in PBS (pH 7.4) containing 0.1% BSA, cover-slipped and examined under a Zeiss epi-fluorescence microscope, and photographed. Control sections were either incubated with non-immune goat or mouse serum or did not receive enzyme pretreatment or exposure to first antibody. The controls in each case showed no specific localization of fluorescein or rhodamine-labeled secondary antibodies. Monkey ear sections were stained in parallel as positive controls.

Cell Culture

For initiation of monolayer culture, the disc was isolated from the PA and further divided under a dissecting microscope into separate regions (AB, IZ, and PB). Regions of the disc and PA were kept separate for all analyses. Disc and PA tissues were minced into approximately 0.5-mm pieces, washed in complete alpha minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and antibiotics (Gibco), and placed into 60-mm Petri dishes containing 3 mL of Vitrogen (Collagen, Palo Alto, CA). This technique has proven remarkably successful in the lab in culturing a wide variety of tissues. The rationale for selecting this modified explant technique for

establishing primary cultures and additional details are provided in Mills.[23] Briefly, the gels were prepared according to the manufacturer's directions. The complete gel contains 2.5 mg/mL type I collagen, 1× alpha MEM, and 10% FCS. Tissue pieces were placed approximately 1 cm apart, the collagen was allowed to gel, and it was immediately overlaid with complete alpha MEM. Explants were incubated at 37°C in 95% humidified air and 5% CO_2. The media from the top of the gels were changed every other day. Disc and PA cells migrate outward from the explant into the three-dimensional matrix with migration rates dependent on tissue type. Posterior attachment explants (fibroblast being the dominant cell type) had a significant outgrowth of cells after several days in culture. Explants derived from band areas and IZ (predominantly chondrocytes) had a "halo" of cells surrounding them in about 10 days. The gels were then washed in BSS, the explant pieces were removed, and the migrant cells were enzymatically liberated by digesting gels with 0.4% collagenase (type CLS III, Cooper, Malvern, PA) for 30 minutes at 37°C. Cells were collected by centrifugation (1,700 g) for 5 minutes, washed in complete alpha MEM, and counted and seeded to form monolayers at 2×10^5 on 60-mm tissue culture dishes (Falcon Plastics, Oxnard, CA). The medium was changed daily until the monolayers became nearly confluent. Only primary cultures were used for proteoglycan analysis.

Labeling and Extraction of Proteoglycans

Regions of the disc were minced into 0.5-mm pieces, placed into organ culture, and labeled for 18 hours with [^{35}S]-sulfate (10 μCi/mL) (New England Nuclear, Boston, MA) in alpha MEM supplemented with 10% FCS and antibiotics. Monolayers, after 3 to 5 days in culture and visual subconfluency, obtained as described above, were labeled in a similar manner. Proteoglycans from the tissues, media, and cell layers were extracted with an equal volume of 8 M guanidine hydrochloride (GuHCl) to achieve a final concentration of 4 M GuHCl.[45] The 8 M GuHCl contained the protease inhibitors (0.1 M 6-aminocaproic acid, 0.1 M Na_2EDTA, 0.005 M benzamide hydrochloride, 0.001 M phenylmethylsulphonlyfluoride, 0.1 M sodium acetate at pH 6.2). After incubation for 24 hours at 4°C, any debris was removed by centrifugation, and the proteoglycan-containing supernatant was saved for further analysis.

Quantification of Proteoglycans

To determine the quantity of proteoglycan present in each sample, an aliquot of each sample was chromatographed on Pharmacia PD-10 columns (Sephadex G25, Pharmacia, Piscataway, NJ) in dissociative buffer (4 M GuHCl). Fractions (0.5 mL) were collected and counted in a scintillation counter. The amount of incorporated label in the void volume fraction is an estimate of the sulfate-labeled proteoglycans present in each sample.

Determination of Proteoglycan Hydrodynamic Size

The relative molecular size of extracted proteoglycans was assessed by placing an aliquot of the 4 M GuHCl extracted sample on a molecular sieve Sepharose Cl-2B column (Pharmacia) that was precalibrated with sulfate-labeled proteoglycans extracted from bovine nasal septum, primate articular cartilage organ culture, and human foreskin-fibroblast (4 day) cell culture. The V_{total} of each chromatographic analysis was determined by inclusion of [^{3}H]-uridine with the sample. The radioactive fractions (0.5 mL) collected were counted in a liquid scintillation counter. The position of the radioactive peaks relative to a known standard will give an accurate estimate of molecular size.

Determination of Chondroitin Sulfate-Dermatan Sulfate

The labeled proteoglycans were dialyzed against water for 72 hours to remove free sulfate and guanidine. The dialyzed proteoglycans were lyophilized and dissolved in 10 mM tris, pH 7, containing 400 μg/mL pronase. Pronase digestion was carried out at 55°C for 24 hours followed by cooling and 5% TCA precipitation.

The supernatant was dialyzed, lyophilized, and passed through a Sephadex G-50 column. The void volume radioactive GAGs were concentrated by ethanol precipitation and dissolved in a minimum volume of buffer (25 mM tris, pH 8). Either chondroitinase ABC or AC was added to the GAG solution (1 unit/mL) and incubated for 4 hours at 37°C. The entire sample was then passed through a Sephadex G-50 column (1 × 20 cm, Pharmacia). The radioactivity of each fraction (1 mL) was determined by scintillation counting.

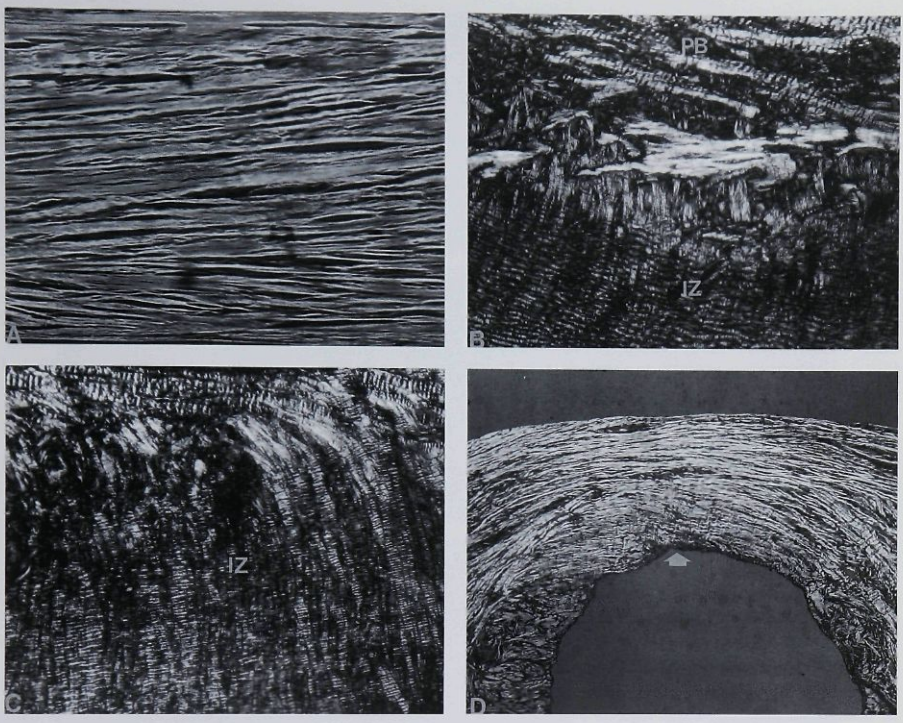

Fig 2 Collagen fiber patterns in the TMJ disc. (A) Parasagittal section through the IZ. Note lateral branching between adjacent collagenous fiber bundles (subadult *Papio*, × 210, CPL). (B) Horizontal section through IZ at the junction with the PB midway between the condylar poles. The collagenous fiber bundles here are interwoven and the continuity between the IZ and PB fibers is not apparent. The crimp in the IZ and posterior part of the PB appears to be greater than at the junction between the two regions (*Macaca*, × 210, CPL). (C) Horizontal section through IZ of disc at its junction with the PB. Here the anteroposterior collagenous fiber bundles of the IZ turn to join those of the transverse fibers of the PB (top of photograph) (*Macaca*, × 47, CPL). (D) Horizontal section through the disc showing the circumferential confluence of the transverse fibers of the AB and PB around the medial side of the IZ (anterior is to the right). Only a portion of the IZ (arrowhead) is apparent in the section (*Macaca*, × 47, CPL).

Results

Histology of the TMJ Disc and Posterior Attachment

Fiber Organization in the Disc. A complex collagenous fiber architecture is revealed by polarized-light microscopy, with regional differences observed in gross pattern and relative compactness of fasciculi. Collagenous fiber branching frequency and crimp also seemed regionally differentiated.

Collagen architecture in the primate TMJ disc is dominated by two collagenous fiber bundle systems. In the IZ, the disc consists of coarse, densely packed collagenous fiber bundles oriented in a predominantly anteroposterior direction, with much lateral branching (Fig 2, A). The density appears greater than in band regions. In horizontal sections, the arrangement of these fiber bundles is more readily demonstrable, traveling in a predominantly anteroposterior orientation in the center of the IZ and arching, inward and outward, toward the medial and lateral poles of the disc (Fig 2, B). At the junction with the band areas, the collagenous fiber bundles arch mediolaterally to become continuous with the transversely oriented fasciculi of the

band regions (Figs 2, B and C). The collagenous fibers of the IZ have the crimp pattern that is characteristic of tendon (Fig 2, A).

The band areas have a significantly more complex network of fiber groups than does the IZ. In both the AB and PB, the transverse collagenous fiber bundle system, oriented at right angles to the collagenous fibers of the IZ, is the predominant fiber group. This mediolaterally directed fiber system encloses the IZ in a roughly circumferential manner (Fig 2, D). Additional fiber bundles travel obliquely through the center of the AB and PB. Collagenous fiber crimp was less conspicuous at the junction of the band areas with the IZ, especially in the PB. In the PA, collagenous fibers are loosely organized in a roughly parallel anteroposterior pattern.

Staining with a modified Verhoeff's elastic stain demonstrated the presence of mature elastic fibers in all regions of the disc and PA. Mature elastic fibers are a prominent component in both the AB and PB, and they consist of a system of fairly straight, small caliber fibers that are oriented more or less parallel with the collagenous fiber bundles (Fig 3, A and C). Elastic fibers lace through the collagenous fiber bundles, branch freely at acute angles, and anastomose with each other (Fig 3, A and C). In the IZ, the straight elastic fibers again parallel the collagenous fiber framework, but they do not branch as frequently and appear to be fewer in number than in the band regions (Fig 3, B). In horizontal sections, a mediolaterally oriented band of elastic fibers is concentrated at the junction of the PB with the PA (Fig 3, D). Elastic fibrils in the PA are of a much larger caliber and are concentrated within its superior lamina (Fig 3, E).

Cell Type and Glycosaminoglycan Distribution. Collagenous fiber bundles in the disc delineate narrow regions containing numerous large cells that display a typical chondrocytic morphology: rounded nuclei surrounded by a large halo of cytoplasm (Fig 4, A to C). Chondrocytes are found distributed throughout the disc but appear most numerous in the center of each band area (Fig 4, A and C). The majority of these cells are found either as paired cell nests or clusters of three to six cells as seen in hyaline cartilage (Fig 4, A and C). In the IZ, chondrocytes are fewer and are aligned in rows paralleling the collagenous fiber bundles (Fig 4, B). Chondrocytes within the disc do show variations in size and shape, being generally smaller and less chondrocytelike at the articular surfaces and toward the attachment areas (Fig 4, D). Chondrocytes are absent from the PA and fibroblastic cells predominate (Fig 4, E).

The stain toluidine-blue is a sensitive indicator for detecting the presence and distribution of sulfated GAGs. Metachromatic staining, indicative of the presence of sulfated GAGs, is present in elevated amounts in the center of each band area, along their inferior condylar surfaces, and to a much reduced extent in the IZ (Fig 4, A to C). In band regions, very intense metachromasia is found in the pericellular matrices surrounding each chondrocyte, with a moderately stained sulfated matrix extending outward from each cell into the adjacent interfibrillar spaces to surround the collagenous fiber bundles (Fig 4, A and C). The PB appears to have slightly greater amounts of sulfated GAGs than does the AB, with metachromatic staining fading at the junction of the PB and PA. There is no evidence of metachromasia in the PA (Fig 4, D).

Proteoglycan Analysis

Cell Culture. Cells grew out from the explants at a steady rate and by 7 to 10 days were ready for establishment as monolayer cultures. Cells from the disc and PA grew freely and thrived on plastic surfaces, reaching subconfluency after 3 to 5 days. This was generally characteristic of all explants. Phase contrast micrography showed that chondrocytes derived from band areas and IZ, and fibroblasts derived from the PA maintained their distinct morphologies in primary culture. Cells from the disc are polygonal in monolayer culture (Fig 5, A) and appear identical to authentic chondrocytes isolated from bovine nasal septum or primate tibial cartilage. Cells from PA explants are flatter and fusiform to spindle-shape, resembling typical fibroblasts (Fig 5, B). All biochemical analyses were performed on primary cultures.

Proteoglycan Hydrodynamic Size Determination. The disc was finely dissected into the AB, PB, IZ, and PA, and it was labeled in complete medium supplemented with [^{35}S]-sulfate for 18 hours. The proteoglycans were extracted and resolved on Sepharose C1-2B columns. Each region of the disc synthesized a high molecular weight proteoglycan similar in hydrodynamic volume to proteoglycans obtained from bovine nasal septum or primate articular cartilage organ cultures, which suggested that there are no regional differences in synthetic ability among regions of the disc (Fig 6). Analysis of [^{35}S]-sulfate–labeled proteoglycans extracted from primary cultures of all three regions of the disc revealed elution profiles that matched in hydrodynamic volume those produced by disc organ cultures, which indicated

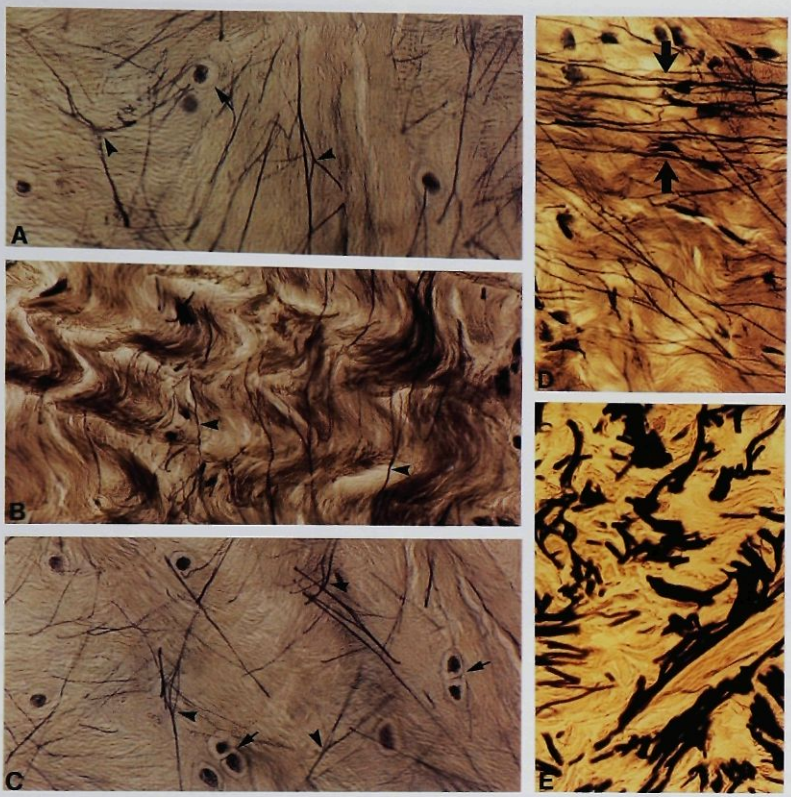

Fig 3 Elastic fiber pattern in the primate disc and PA. Small caliber elastic fibers are prominent in both the anterior (A, parasagittal section) and posterior (C, parasagittal section) band regions and generally parallel or are partly intercrossed with the collagenous fiber bundles. Elastic fibers are seen to frequently branch diagonally between other elastic fibers (arrowheads). In the IZ, the elastic fibers also parallel the collagenous fiber bundles but do not branch or anastomose as frequently (B, parasagittal section). Note also how the elastic fibers appear to bisect the crimp (wave) pattern formed by the collagenous fiber bundles (arrowheads). A concentration of mediolaterally oriented elastic fibers (defined by large black arrows) are observed at the junction of the PB and PA (D, horizontal section). Elastic fibers are much thicker and more prominent in the superior lamina of the PA (E). Black arrows identify chondrocytes. (Modified Verhoeff's elastic stain, × 25.)

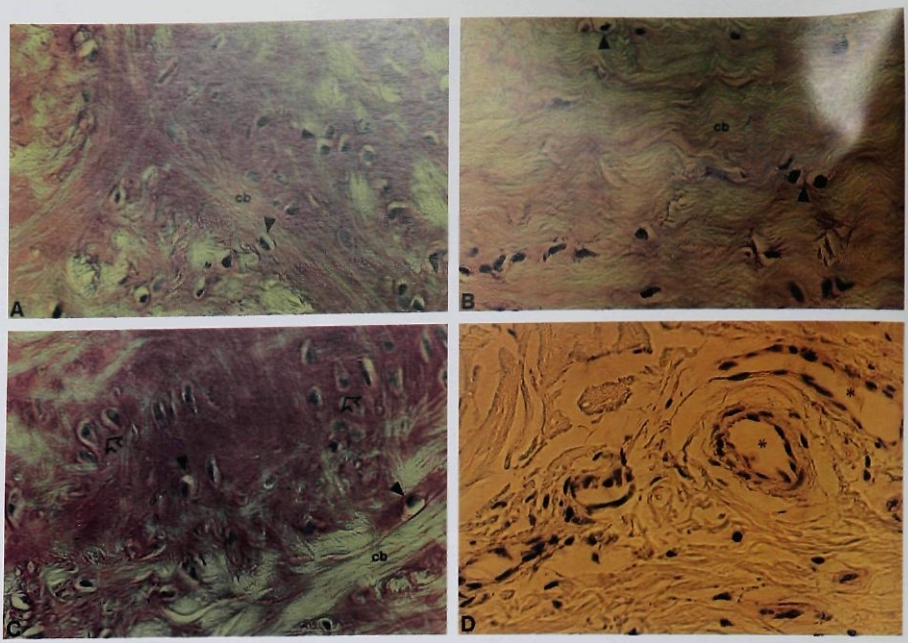

Fig 4 Cell morphology and pattern of GAG localization in the primate disc and PA. The primate disc consists of numerous cells with a morphology typical of hyaline cartilage cells: rounded nuclei with a large surrounding halo of cytoplasm (arrowhead). Chondrocytes are found throughout the disc, many found in cell clusters (open arrow), within the interfibrillar spaces formed by the collagenous fiber bundles (cb). Cells are most numerous in the band areas, where they are found typically in cell nests or cell clusters of four to six cells (A and C). In the IZ, chondrocytes are fewer and aligned in columns paralleling the collagenous fiber bundles (B). The PA does not contain chondrocytes but is populated mostly by fibrocytes (D). Toluidine-blue staining, indicative of the presence of sulfated GAGs, is present in significant amounts in the center of each band area and to a much reduced extent in the IZ (A to C). In band regions, intense metachromasia is found in the pericellular matrices surrounding each chondrocyte with a moderately stained sulfated matrix extending outwards from each cell to fill the adjacent interfibrillar spaces formed by the collagenous fiber bundles (A to C). The PB appears to have slightly more elevated amounts of sulfated GAGs than the AB (compare C with A). Metachromatic staining fades toward the junction of the PB and PA. There is no evidence of metachromasia in the PA (D). Asterisks identify blood vessel lumina (parasagittal section, toluidine-blue stain, × 25).

that the disc's chondrocytic phenotype is maintained in monolayer culture (Fig 6). Proteoglycans extracted from PA organ and monolayer cultures synthesized a much smaller population of proteoglycans, which had a hydrodynamic volume similar to human foreskin fibroblast proteoglycan (Fig 6).

Total [^{35}S]-sulfate incorporation into macromolecules as evidenced by PD-10 chromatography indicated that the chondrocytelike cells from the disc synthesized approximately 6 times more labeled proteoglycan than did fibroblastlike PA cultures, and band areas generally incorporated more sulfate into proteoglycan than the IZ.

Determination of GAG Chain Composition. Glycosaminoglycan chains were released from labeled proteoglycans and analyzed on sephadex G-50 columns before and after chondroitinase ABC or AC digestion. The labeled GAGs derived from disc cell organ and monolayer cultures were almost completely digestible with chondroitinase ABC and AC (Table 3), which was consistent with their being predominantly chondroitin sulfate. Posterior attachment proteoglycans were digestible with chondroitinase ABC but were only partially digestible with AC, which was consistent with their being predominantly dermatan sulfate (Table 3).

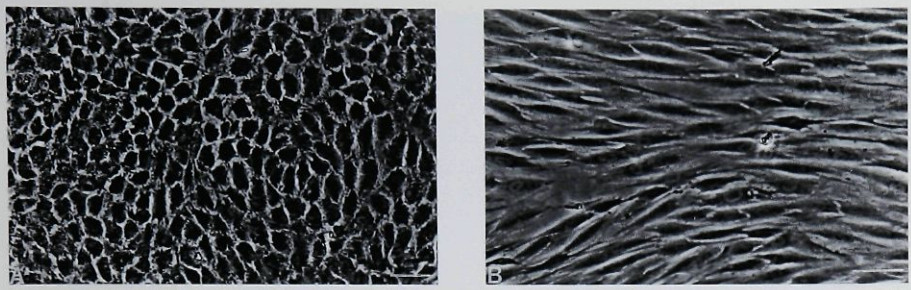

Fig 5 Morphology of disc and PA cells in primary monolayer culture. Phase contrast photography demonstrates that the characteristic chondrocytic morphology of disc cells (A, posterior band) and fibroblastic shape of posterior attachment cells (B) are maintained in monolayer culture. Phase-contrast photomicrographs were taken on day 3 in culture. (Bars = 50 μm.)

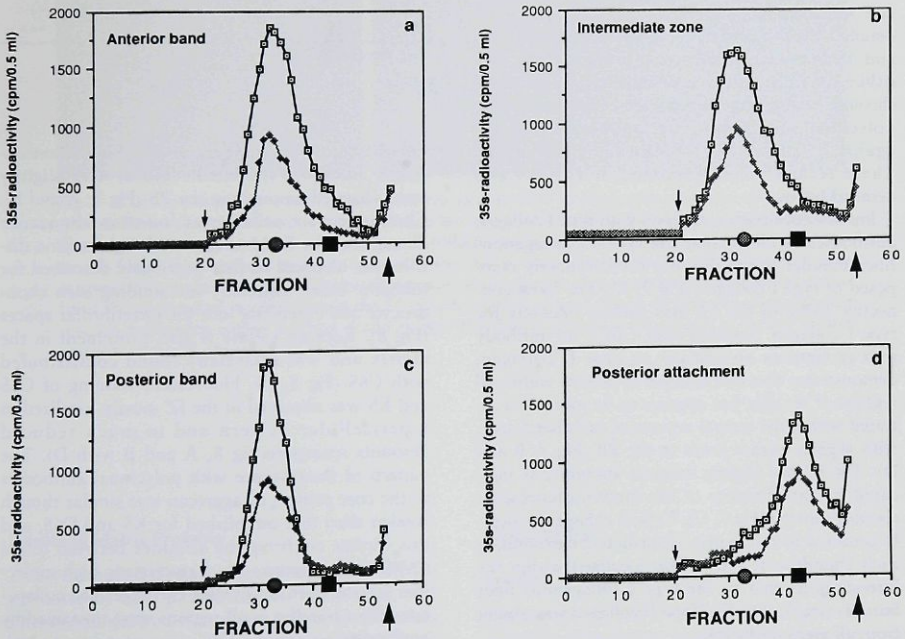

Fig 6 Proteoglycan profiles from organ and monolayer culture of disc and PA. The 4 M GuHCl extractable proteoglycans from disc and posterior attachment were chromatographed on an analytical Sepharose Cl-2B column eluted with dissociative buffer. Black line shows the elution profile of newly synthesized [^{35}S]-sulfate–labeled proteoglycans from monolayer cultures. Dotted line shows the elution profile of proteoglycans obtained from organ culture. Black circle indicates the elution position of bovine nasal and primate articular cartilage proteoglycan, black square shows the elution position of human foreskin fibroblast proteoglycan. (Small arrow = included volume, large arrow = void volume; a = anterior band cultures, b = intermediate zone cultures, c = posterior band cultures, d = posterior attachment cultures.)

Table 3 Percent GAGs Digested by Chondroitinase ABC or ACII

Region	ABC*	ACII**
PB	96	94
AB	94	97
IZ	95	96
PA	92	61

*Chondroitinase ABC specifically degrades chondroitin-four sulfate, chondroitin-six sulfate, dermatan sulfate, and hyaluronic acid.
**Chondroitinase ACII degrades all of the above except dermatan sulfate.

Table 4 Immunolocalization of Cartilage GAGs, Core Protein, and Types I and II Collagen in the Primate Disc

Antibody	Positivity
Glycosaminoglycans	
3-B-3	+++
9-A-2	++
CS56	++
5-D-4	+++
Chondroitin-sulfate Proteoglycan	
GpCP	+
RaCP	+
Type I collagen	
GpIC	++
SPI	++
Type II collagen	
GpIIC	++
CIICI	++
Cartilage matrix products	
1-B-5	++
8-A-4	++
SFN	+++

+++, strongly positive; ++, positive; +, weakly positive.

Immunohistochemistry

Collagens. Light-microscopic studies and analyses of extracted proteoglycans from all regions of the disc established that the disc has a cartilagelike matrix. These analyses do not, however, allow precise localization of matrix constituents. A more detailed level of analysis to define the distribution and arrangement of collagens, proteoglycans, and other cartilage matrix proteins was initiated through indirect immunofluorescence employing polyclonal and monoclonal antibodies directed against a variety of cartilage matrix products. These results are summarized in Table 4 and detailed below.

Immunolocalization analyses with type I collagen antibodies establish that the coarse, collagenous fiber bundles in the disc are predominantly composed of type I collagen (Fig 7, A). The loose connective tissue of the PA also stained intensely for type I collagen. Staining with CIICI, an antibody that recognizes an epitope on type II collagen, demonstrates that this collagen is present within all regions of the disc but appears to be more concentrated within the central regions of each band area, with slightly higher levels in the PB (Fig 7, B and D). The IZ has slightly reduced amounts, as indicated by the intensity of the immunofluorescent staining pattern (Fig 7, C). Type II collagen is mostly confined to the matrix immediately surrounding each chondrocyte but is also localized within the interstices formed by the type I collagenous fiber bundles (Fig 7, B to D). Type II collagen was absent from all areas of the PA.

Proteoglycans. While the biochemical analyses establish that all regions of the disc predominantly synthesize CSPG, comparative immunolocalization analyses demonstrate that discal CSPG has a unique regional pattern of distribution within the primate disc. Indirect immunofluorescence staining with monoclonal antibodies that specifically recognize chondroitin-six sulfate (C6S) and keratan sulfate (KS) demonstrate that these GAGs are concentrated mainly in the band regions, with slightly more elevated amounts in the PB (Fig 8, A and B). Chondroitin-six sulfate was found in abundance throughout the ECM of the band regions (in a distribution identical to that previously described for toluidine blue), especially surrounding each chondrocyte and extending into the interfibrillar spaces (Fig 8). Keratan sulfate is also prominent in the matrix and was essentially found codistributed with C6S (Fig 8, C). Fluorescent staining of C6S and KS was observed in the IZ mostly localized in a pericellular pattern and in much reduced amounts (compare Fig 8, A and B with D). The pattern of fluorescence with polyclonal antibodies to the core protein of aggrecan was similar though weaker than that established for KS and C6S, and this further confirms the affinities between discal CSPG and aggrecan, the characteristic high molecular weight CSPG in hyaline cartilage. The collagenous fiber bundles in all regions were unstained by antibodies to either CSPG core protein or cartilage-specific GAGs.

Immunostaining with monoclonal antibodies to chondroitin-four sulfate (C4S) showed that this GAG is also present in the ECM of the disc. The staining for C4S is similar to that obtained with C6S and KS antibodies (Fig 9, A). Cartilage-characteristic GAGs and CSPG core protein were not found in the PA.

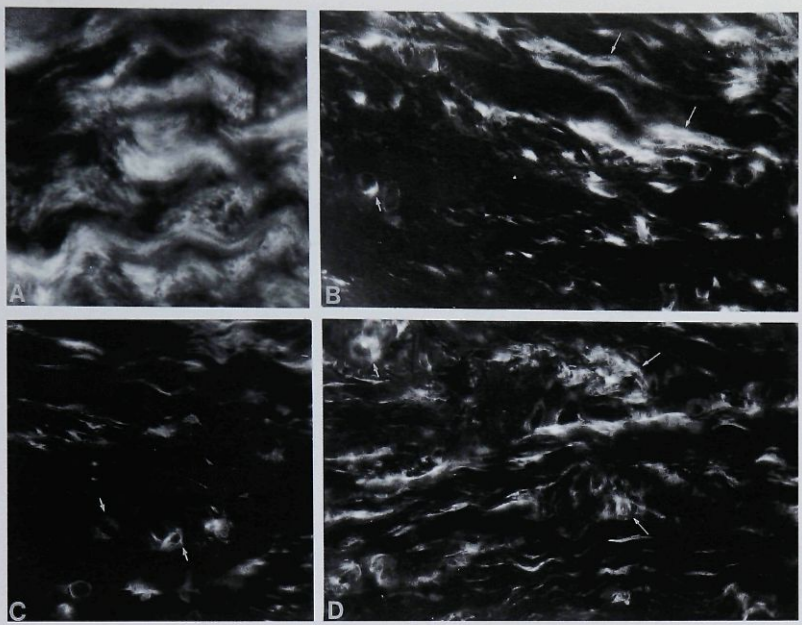

Fig 7 Distribution of type I and II collagens in the primate disc. Staining with antibodies to type I collagen establishes that the numerous coarse collagen fiber bundles within the disc are composed primarily of type I collagen (A, parasagittal section). Type II collagen is also a significant component in the disc. It is mostly found in pericellular matrices (short arrows), but is also present in the interstitial matrices (long arrows). It is present in significantly greater amounts in the anterior (B, parasagittal section) and posterior band (D, parasagittal section) than in the IZ (C, parasagittal section). Based on intensity of the staining reaction (indicating greater reaction product), type II collagen appears to be present in slightly greater amounts in the PB. (Parasagittal section, × 10.)

Cartilage Matrix Proteins

Assay through indirect immunofluorescence for the presence of link protein, hyaluronic acid, and fibronectin showed that these matrix proteins are prominent components of the disc's ECM. Link protein and hyaluronic acid were distributed throughout the ECM but localized most intensely in the band areas and to the matrices directly surrounding each cell (Fig 9, B and C). This staining pattern is quite similar to that previously established for CSPG core protein and GAGs. Fibronectin is mostly associated with the collagenous fiber bundles in both the disc and PA (Fig 9, D).

Discussion

Loading of the TMJ

The notion that the jaw joint should not be subjected to compressive loading during jaw function has been around for a long time. This idea appeared in a refereed journal as late as 1986.[46] Reviews of the subject by Hinton[47] and Hylander,[48] as well as recent modeling studies[49] and loading experiments,[50] indicate that there is considerable indirect and cogent direct evidence that the jaw joints are indeed loaded during function. The present study regards this as fact.

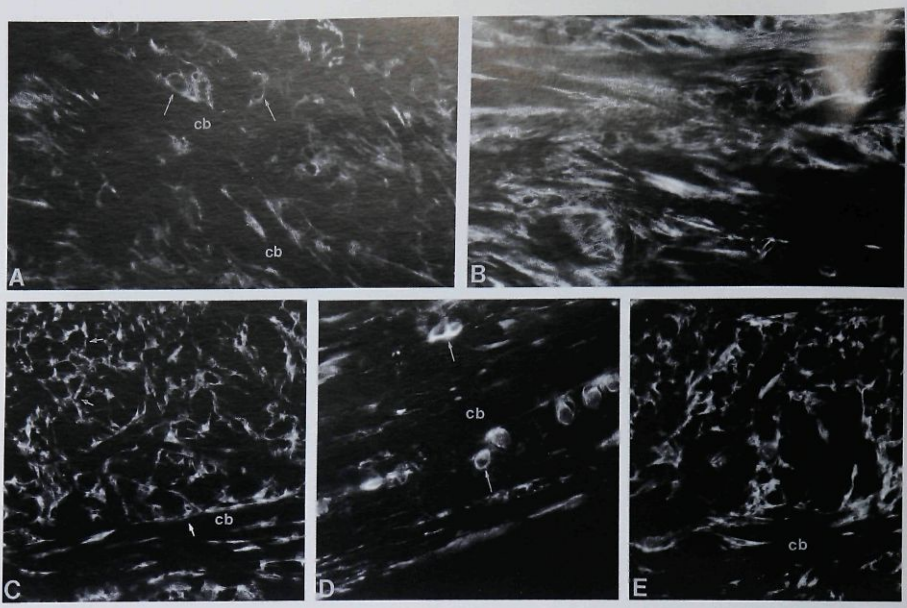

Fig 8 Localization of cartilage-specific GAGs in the primate disc. Immunostaining with the antibody 3-B-3 (identifies chondroitin-six sulfate) demonstrates that this GAG is a prominent component within the interstitial matrices formed by the collagenous fiber bundles (cb) in the anterior band (A) and posterior band (B). It is most intense around each chondrocyte (arrows) and is present in much reduced amounts in the intermediate zone (D, compare D with either A or B). Based on intensity of the staining reaction (an indication of greater reaction product), CSPG is present in greater amounts in the posterior band. Staining with 5-B-4 (recognizes keratan sulfate) shows a pattern of immunolocalization identical to that of chondroitin-six sulfate (C). E is a section taken slightly deeper to that represented in C and is stained with antibody 3-B-3. Arrows in C identify collagenous fiber bundles in cross section. (Parasagittal section, × 10.)

Content of the ECM

The intensely birefringent fasciculi that dominate the ECM of the disc are predominantly composed of type I collagen. This finding is consistent with that of previously published work.[23,24,51] Type I collagen was also present in the PA, where it is much less compactly arranged than in the disc.

Type II collagen was also demonstrated in the disc. This finding conflicts with the observations of Milam et al[24]; however, type II collagen has been demonstrated in the disc of other mammalian species[26,52] and in other load-bearing fibrocartilages.[7,53–55] Since immunoreactions are determined by several factors—purity and specificity of the antibody, unmasking and exposure of the epitope, the concentration of the fixative, demineralization processes, etc—the failure of Milam et al to demonstrate type II collagen is probably attributable to the vagaries of tissue processing, causing in this case the loss of antigenicity.

In general, type II collagen staining was most intense pericellularly but was also present in the interterritorial matrix that extends into the interstices of the type I collagenous fiber bundles. Also, the staining was most prominent in the band regions and notably less intense in the IZ. Type II collagen was not found in the PA.

The glycosaminoglycans C6S, C4S, and KS were present in apparent codistribution throughout the disc. Each was more concentrated in the band regions, with lesser representation in the IZ, and generally paralleled the intensity of localization of type II collagen. Staining with antibodies to aggrecan core protein, link protein, and hyaluronic acid further established the hyaline cartilagelike matrix of the disc. Cartilage matrix proteins were not found in the PA.

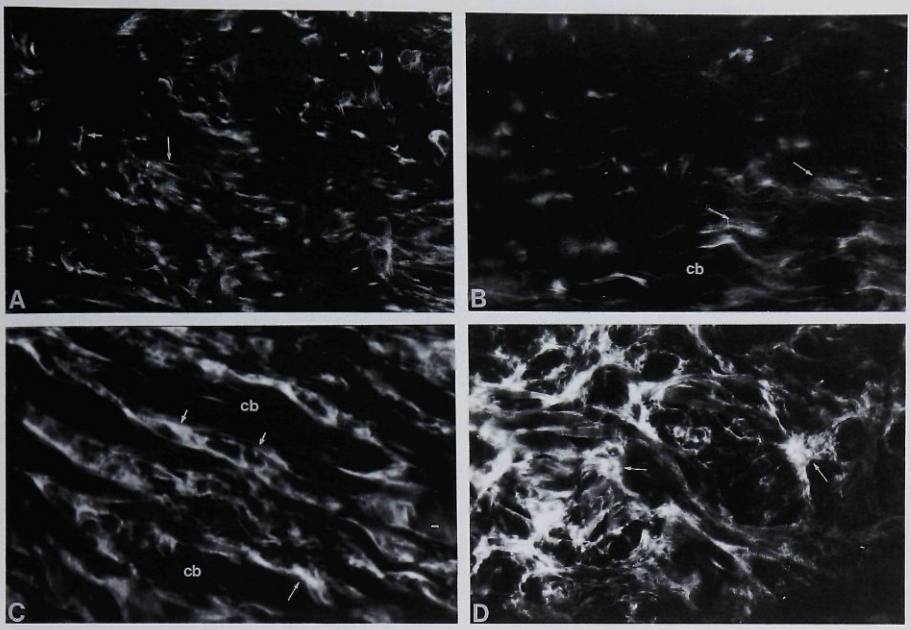

Fig 9 Identification of cartilage matrix products in the primate disc. Chondroitin-four sulfate is present in a staining pattern virtually identical with the pattern for KS and C6S: very intense pericellularly (arrowheads) but also present throughout the interstices of the collagenous fiber bundles (arrows) (cb) (A, posterior band). Link protein (B, inferior surface of anterior band) and hyaluronic acid (C, posterior band) are additional components of the disc's ECM and are found distributed in both pericellular (arrowheads) and interstitial matrices (arrows). Fibronectin is present throughout the disc and is intimately associated with the type I collagenous fiber bundles (D, intermediate zone). Collagenous fiber bundles (cb) in C are cut in cross section. (Parasagittal section, × 10.)

Elastic fibers were present throughout the disc and the PA, the major distinction between these sites being that the caliber of fibers was notably larger in the latter.

According to its contents then, the ECM of the disc is an elastic fibrocartilage.

Properties of Cartilage

Since direct investigation of discal properties has been limited (see below), inferences about the physical properties of the disc must be drawn by analogy with hyaline cartilage or fibrocartilages, about which more is known.

Hyaline Cartilage

The mechanical properties of cartilage are directly related to its matrix composition and architecture.[9,56–59] In the superficial zone of articular cartilage, proteoglycan concentration is low and collagen content is high (70% to 80% dry weight), and collagenous fibers are arranged parallel to the surface.[60–62] In the midzone, the collagenous fibers are larger in diameter and randomly oriented. In the deep zone, collagen content decreases to 60% and proteoglycan content increases. The fiber bundles are largest here, are oriented perpendicularly to the surface, and are anchored in calcified cartilage, which is in turn anchored on the subchondral bony plate. The proteoglycans are mechanically entrapped within the interstices of the collagenous fibers and have electrostatic interactions with them. The high negative charge of the entrapped proteoglycans generates a high Donnan osmotic pressure differential between the interstitial fluid of the ECM

of the cartilage and the synovial fluid that bathes it.[63–65] The result is that the ECM has a great affinity for water and thereby generates a fluid swelling pressure that distends the collagenous framework, and therefore the cartilage, from within.

Application of a load to the articular surface compresses the cartilage and compacts the collagenous fiber-proteoglycan mesh. The superficial collagenous fibers, by virtue of their parallel relation to the surface, act as a tension-resistant diaphragm[63,64] that distributes the load across the surface of the cartilage and, via connections with subjacent collagen, to the subchondral plate. Fluid within the cartilage becomes pressurized and passes through the cartilage surface and laterally between the pores of the collagenous fiber-proteoglycan mesh. Expression of fluid through the surface is countered by the osmotic pressure, and lateral flow through the ECM is resisted by frictional drag of the fluid on the compacted collagenous fiber-proteoglycan mesh. Compaction of the mesh also concentrates the negatively charged proteoglycans, which increases the repulsive force between these like-charged molecules. These mechanisms are responsible for most of the compressive stiffness of cartilage.

Knee Meniscus Fibrocartilage

The jaw joint disc has certain features comparable to hyaline articular cartilage, but it is compositionally and structurally more similar to knee meniscus. The disc and meniscus[63–67] both are dominated by large, type I collagenous fiber bundles, contain reduced amounts of type II collagen and proteoglycans, and have elastic fibers in the ECM. While lesser in proportion, the proteoglycans are similar to those of hyaline cartilage and their GAG profiles are also comparable. The organization of the surface collagenous fibers appears to be identical. The organization of collagenous fibers in the depths of the ECM is also similar in that large, intimately associated fasciculi have mutually perpendicular orientations. The expectation of these several similarities is that the material properties of knee meniscus and disc should be roughly comparable.

Properties of the Disc

Relatively little direct investigation of discal properties has been done, and none has been done on intact discs. The disc has been shown to have viscoelastic properties under compressive loading[32] and, as might be expected, differential tensile stiffness that is a function of collagenous fiber orientation, ie, the stiffness is greater when tension is applied in line with the predominate collagenous fiber axis and lowest when applied across it.[33,34] Stress relaxation of discal tissues is much less than in hyaline cartilage.

These findings are consistent with observations on knee meniscus. Compared to hyaline cartilage, knee meniscus, when tested along the axis of its circumferential fibers, vis à vis the axis of the split lines in articular cartilage, has about 10 times greater tensile stiffness. Conversely it has much less stiffness in compression and dynamic shear than articular cartilage. It also has notably less permeability, ie, greater resistance to fluid flow through the ECM.

The mechanical properties of the meniscus make it readily deformable and conformable to the contours of the femoral articular surface. This thereby provides for excellent shock absorption and promotes the distribution of compressive loads.

Since knee meniscus and jaw joint disc are compositionally and structurally similar, it is expected that the disc would possess similar biomechanical properties and biologic functions. However, the tissue coverings of the articulating bones in the knee and jaw joint differ. In the knee, the articular tissue is hyaline cartilage; in the jaw joint, it is fibrocartilage, which is compositionally comparable to the jaw joint disc. Again reasoning by analogy with the knee meniscus, the articular tissues of the jaw joints should be relatively more deformable and mutually more conformable under load than joints with articular coverings of hyaline cartilage.

Placing these inferred properties in the context of jaw function leads to the conclusion that the congruity between the articulating structures—the temporal bone, disc, and condyle assembly—should be relatively greater than in joints with hyaline articular coverings. The functional import of increased congruity is that it promotes joint stability at all condyle/disc positions. This would seem to be important in a jaw joint, in which the amount of relative movement between the condyle and disc varies notably depending on the specific jaw movement, and in which the loading regimen may vary significantly in magnitude and direction. For example, compare hard biting at the molars with the jaws nearly closed vis à vis the incisors with the jaws widely opened.

The distribution of proteoglycans is not homogeneous in the meniscus[66] nor, as the present study shows, in the jaw joint disc. The proteoglycan content appears greatest in the PB and least in the IZ

of the disc. Proteoglycan content is correlated with permeability and compressive stiffness.[65] Therefore, the PB of the disc, by virtue of its apparently greater proteoglycan content, ought to have the greater resistance to compressive loads than other disc regions. Also the essentially mutually perpendicular orientation of the type I collagenous fiber bundles at the junction of the IZ and PB may reflect, as in knee joint meniscus,[65] the development of "hoop" stresses during loading. If these observations are valid, then several corollary inferences on condyle-disc control mechanisms and loading regimens are suggested.

The presence of elastic fibers in the mammalian jaw joint has long been known.[17,19,29,68–70] Elastic fibers are found in many types of connective tissues. In general, they offer only slight resistance to elongation[71] but in some arrangements they may support noteworthy loads. For example, the elastic ligamentum nuchae of ungulates is formed of densely packed, large caliber elastic fibers and relatively small amounts of collagen.[72,73] In the camel it is able to maintain head posture by itself.[74] However, in the disc, the elastic fibers are of small caliber and much less densely packed than in the ligamentum nuchae and probably are not likely to develop tensile stress sufficient to support much of the joint load.

While elastic fibers appear to be present in minor numbers in hyaline articular cartilage,[75] the high Donnan osmotic pressure and the swelling pressure that attends it would appear to be responsible for restoration of the form of load-compressed cartilage after load removal. Since swelling pressure is a function of proteoglycan content, and the latter is much lower in the disc than hyaline cartilage, the restorative swelling pressure in the disc would be expected to be much reduced. Therefore, elastic fibers may be more important in restoration of disc form after load removal and in the maintenance of resting disc shape than they are in the restoration of resting form of articular cartilage—the elastic recoil of the fibers compensating, as it were, for the low proteoglycan content. That the elastic fiber system is under at least some tensile stress in the resting disc is suggested by its generally rectilinear microscopic appearance in association with crimped collagenous fiber bundles (Fig 3). The idea that the recoil of elastic fibers may be involved in the maintenance of disc form has been around for a long time,[19,68,69,76] but the requirement for their conspicuous inclusion in the jaw joint disc, vis a vis their paucity in hyaline articular cartilage, as a function of low proteoglycan content has not been apparent before.

Properties of the Posterior Attachment

The noncompact arrangement of type I collagenous fibers in the PA as well as the lack of type II collagen and CSPG indicate that this tissue should not be stiff. These observations are consistent with the known capacity of the attachment in monkeys and humans to undergo large volumetric fluctuations.[77–80]

Special Disc Functions

While the composition of the jaw joint disc may be generally similar across mammalian species, the construction of the jaw joints in this class of animals varies considerably, far more than knee joints for example. Also, the disc may assume special functions in certain species. For example, in the giant panda, which has an enormous biting capacity, the disc is paper thin.[81] In the shrew, *Sorex minutus*, the condyle has separate superior and inferior articular surfaces, but the disc covers only the superior facet and is extended inferiorly from it over a nonarticulating area to fuse with the bone adjacent to the articular covering of the lower joint surface.[82] In canids, the disc appears to constrain lateral jaw movement along a plane appropriate for carnassial tooth function.[83] The disc is absent in some species.[76,84] Numerous other examples of jaw joint diversity could be cited. The point about the diversity is that, while the general properties of the disc may be the same in mammals, specific functions may differ widely. The above conclusions on primate discs may therefore not have general applicability.

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Resumen

Investigaciones morfológicas, microscópicas e inmunológicas sobre la función del disco primado de la articulación temporomandibular

Se examinó la distribución y disposición de las proteínas de la matriz extracelular en el disco primado de la articulación temporomandibular y en la inserción posterior, utilizando una combinación de microscopio de luz y de técnicas inmunohistoquímicas y bioquímicas. Las áreas correspondientes a las bandas del disco contenían una red compleja de fibras colágenas (tipo I) consistentes de un manojo de fibras dirigidas mediolateralmente, que se entrelazan o que se continúan con un conjunto de fibras colágenas dirigidas anteroposteriormente que corre a través de la zona intermedia. Existen fibras elásticas y finas que se ramifican y son un componente significativo del disco; éstas están generalmente orientadas paralelamente a la red de fibras colágenas. Los espacios interfibrilares en las áreas donde están las bandas, contienen numerosos condrocitos encajados dentro de una matriz rica en proteoglucano — sulfato de condroitín y colágeno tipo II, con un peso molecular alto. La zona intermedia parece ser tendinosa y está compuesta de fibras colágenas y fibras elásticas orientadas anteroposteriormente, de condrocitos esparcidos, y en bajas cantidades contiene proteoglucano — sulfato de condroitín y colágeno tipo II. La inserción posterior está compuesta de fibrocitos, fibras elásticas de gran calibre, fibras colágenas tipo I sueltas, y proteoglucano — sulfato de dermatán de bajo peso molecular. Estos resultados indican que el disco temporomandibular primado es un tejido microheterógeno con especializaciones regionales precisas.

Zusammenfassung

Morphologische, mikroskopische und immunohistochemische Untersuchungen der Funktion des Kiefergelenk-Diskus von Primaten

Gegenstand der Untersuchung bildete die Verteilung und Anordnung von Proteinen der extrazellulären Matrix des Kiefergelenk-Diskus und dessen posterioren Attachments unter Verwendung einer Kombination von Lichtmikroskopie, Immunohistochemie und biochemischen Techniken. Die Bandanteile des Diskus enthalten ein kompliziertes Fasernetzwerk aus Kollagen (Typ 1), das aus einem mediolateral gerichteten Faserbündelsystem besteht und überlappt oder übergeht in ein anteroposterior gerichtetes; Faserbündel, das die intermediäre Zone durchquert. Dünne, verästelte elastische Fasern sind eine für den Diskus typische Komponente und generell parallel zu den kollagenen Fasernetzen angeordnet. Die interfibrillären Räume der Bandanteile enthalten zahlreiche Chondrozyten, die in eine Matrix von hohem Molekulargewicht - vor allem aus Chondroitin-Sulfat Proteoglykan - und Kollagen (Typ II) eingebettet sind. Die intermediäre Zone präsentiert sich sehnig in ihrer Konstruktion und setzt sich aus anteroposterior orientierten kollagenen und elastischen Fasern, zerstreuten Chondrozyten und einer verminderten Menge von Chondroitin-Sulfat Proteoglykan und Kollagen (Typ II) zusammen. Das posteriore Diskusattachment besteht aus folgenden Komponenten: Fibrozyten, dicke elastischen Fasern, locker vereinigte kollagene Fasern (Typ I) und Dermatan-Sulfat Proteoglykan mit tiefem Molekulargewicht. Diese Resultate deuten an, dass der Diskus des Kiefergelenkes von Primaten mikroheterogenes Gewebe unfasst, das deutliche regionale Spezialisationen aufweist.