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Proteoglicanos da cartilagem epifisária de rãs: Composição, estrutura e variações regionais

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Dedico

Índice

I - Resumo

A cartilagem epifisária de *Rana catesbeiana* foi estudada com o intuito de caracterizar aspectos relacionados aos proteoglicanos, em especial ao agrecam. A cartilagem epifisária foi dividida em três regiões, denominadas articular, lateral e de crescimento, que foram analisadas em nível bioquímico e estrutural. A partir de experimentos bioquímicos e imunoquímicos, utilizando diferentes anticorpos contra os domínios G1, G1/G2 e G3 do core protéico, contra os sítios de ligação das cadeias de condroitim sulfato, da análise do padrão de sulfatação das cadeias de condroitim e queratam sulfato e da associação com o ácido hialurônico e proteína link, o grande proteoglicano de rã foi caracterizado e classificado como agrecam, dada a sua semelhança com o agrecam de mamíferos e de aves. O tamanho do core protéico não variou entre as três regiões (~300kDa). A presença dos três domínios globulares foi também confirmada através da ultraestrutura da molécula de agrecam após *rotary shadowing*. Quando analisadas por métodos cromatográficos, as cadeias de condroitim sulfato apresentaram o mesmo tamanho hidrodinâmico (Kav=0.42) nas três cartilagens. Entretanto, na região de crescimento foi detectada uma sub-população de cadeias de condroitim sulfato maiores (Kav=0,35). Estes resultados foram confirmados através da análise dos dissacarídeos saturados e insaturados, que constituem as cadeias de condroitim sulfato, obtidas após digestão das cadeias com enzimas específicas seguidas por análise cromatográfica, que mostrou que as cadeias de condroitim sulfato na região de crescimento, possuem em média 56 dissacarídeos comparados com os 42 estimados para as outras duas regiões. O dissacarídeo ΔDi4S foi predominante em todas as cartilagens e a razão entre Δdi6S e Δdi4S foi 0,4 para as cartilagens articular e lateral e 0,7 para a cartilagem de crescimento. O dissacarídeo insaturado não sulfatado (ΔdiOS) apresenta maior concentração na cartilagem de crescimento. O terminal não redutor (dissacarídeo saturado) predominante nas três cartilagens foi GalNAc4S. A cartilagem lateral e de crescimento apresentaram a mesma razão entre GalNAc4,6S e GalNAc4S foi 0,5, enquanto a cartilagem articular a razão foi 0,7. A região rica em queratam sulfato foi isolada através de tratamentos enzimáticos e cromatográficos e, após análise eletroforética, apresentou uma massa molecular aparente de ~110kDa. Os dissacarídeos repetitivos das cadeias de queratam sulfato foram predominantemente monossulfatados (Gal-GlcNAc6S) e a razão entre os dissacarídeos mono e dissulfatados foi aproximadamente igual para todas as cartilagens

(0,23). Os resultados revelaram a semelhança estrutural entre o agrecam de rãs e o agrecam de mamíferos, além de demonstrar variações nas cadeias de condroitim sulfato entre a cartilagem de crescimento e as demais regiões.

II - Abstract

A large proteoglycan was isolated from the articular, lateral and growth regions of the femoral distal cartilage of the bullfrog, *Rana catesbeiana*, and identified as aggrecan by a series of biochemical and immunochemical assays. The isolated monomer showed a polydisperse behavior on Sepharose CL2B, with a peak at Kav= 0,17. This proteoglycan was shown to contain the three globular domains G1 and G2 (spaced 21nm from each other) and G3, and to aggregate with hyaluronan and bovine link protein. The core protein was prepared after chondroitinase and keratanase digestion and visualized by Western blotting with the aggrecan domain specific antibodies anti-ATEGQV (G1), anti-CDAGWL (G1/G2) and Lec7 (reactive with the lectin-like domain of G3). All three antibodies reacted with a predominant species that migrated with an apparent molecular size of 300kDa. The bullfrog aggrecan was substituted with both chondroitin and keratan sulfate chains. The condroitim sulfate chains showed a Kav of 0.42 after chromatography of all cartilages, but in the growth cartilage a population showed a Kav of 0.35. Aggrecan from articular and lateral cartilages contained concroitim sulfate chains that were composed of an average of 42 disaccharide units and 56 dissaccharide units in the growth cartilage. The ratio between the unsaturate disaccharides (ΔDi6S/ΔDi4S) was 0.4 for articular and lateral cartilage and 0.7 for the growth cartilage and contained a higher proportion of unsulfated and 6-sulfated disaccharides. The condroitin sulfate chains contained mostly either GalNAc4S or GalNAc4,6S at non-reducing terminal in all three regions. Keratan sulfate was also detected on both the intact aggrecan and the isolated KS-rich region. For all three cartilage regions, the disaccharides of keratan sulfate were ~80% Gal-GlcNAc6S and ~20% Gal6S-GlcNAc6S. These data demonstrate the evolutionary conservation of the aggrecan structure in a lower vertebrate and suggest a high dependence of its functions on the domain organization.

III - Introdução

A matriz extracelular consiste em agregados de componentes macromoleculares específicos que resultam em diferentes estruturas com propriedades biomecânicas distintas, variando entre arranjos finos e transparentes como o da córnea e duros e resistentes como o dos ossos. Entre arranjos tão distintos existem ainda inúmeros tecidos que provavelmente constituem um gradiente estrutural e funcional. Esta variedade em estrutura e função parece ser fundamentada nas diferentes formações fibrilares em associação ordenada com componentes não fibrilares. Entre os primeiros situam-se as fibrilas colagênicas, as fibras elásticas e alguns componentes microfibrilares, enquanto que os componentes não fibrilares são representados por proteoglicanos e glicoproteínas não colagênicas.

Na matriz extracelular da cartilagem hialina encontramos fibrilas heterotípicas de colágeno (Mayne & Burgeson, 1987; Van der Rest & Garrone, 1991; Mayne & Brewton, 1995), formadas pelas associação do colágeno do tipo II com moléculas de colágeno do tipo IX e do tipo XI e uma grande quantidade de proteoglicanos. Outros tipos de colágeno podem ser encontrados nas cartilagens. Associações com moléculas do tipo I e do tipo VI (principalmente nas fibrocartilagens) (Wu *et al*, 1987; 1991) e do tipo X (nas cartilagens de crescimento) (Wallis, 1993) são conhecidas. As fibrilas de colágeno II são formadas por um *core* de colágeno tipo XI e decoradas por moléculas de colágeno do tipo IX. Estas últimas estabelecem ligações cruzadas com o colágeno II (Eyre *et al*, 1987; van der Rest, 1988) e entre si, não somente na mesma fibrila mas entre fibrilas adjacentes, colaborando para a estabilização da malha colagênica (Wu *et al*, 1992).

Associados à superfície das fibrilas de colágeno são ainda encontradas moléculas dos pequenos proteoglicanos fibromodulim e decorim, que são capazes de modular a espessura das fibrilas de colágeno formadas *in vitro* (Hedbom & Heinegård, 1989; 1993; Hedlund *et al*, 1994). Parece também que o decorim está envolvido com a fibrilogênese do colágeno *in vivo* (Birk et al, 1995). Além deste controle sobre o diâmetro das fibrilas, têm-se assumido que estes pequenos proteoglicanos são capazes de organizar os espaço interfibrilar e de controlar os mecanismos de fusão de fibrilas (Scott, 1988; Heinegård & Pimentel, 1992; Birk *et al*, 1995)

Sem formar fibras, as fibrilas de colágeno apresentam distribuição organizada e orientada nas cartilagens. Genericamente, na camada superficial existe uma distribuição de fibrilas paralelas à superfície da cartilagem. Imediatamente abaixo desta camada existe uma distribuição menos preferencial das fibrilas que numa região mais profunda distribuem-se perpendicularmente à superfície (Yarker *et al*, 1983; Hukins & Aspden, 1985). Esta distribuição parece se encaixar ao princípio dos arcos Góticos de Benninhoff (Benninhoff, 1925), sendo que este padrão de distribuição da malha colagênica parece ser fundamental ao entendimento das modernas teorias de "consolidação da matriz", proposta para explicar a fisiologia das cartilagens articulares (Oloyede & Broom, 1994).

Ultra-estruturalmente, as fibrilas de colágeno apresentam sempre diâmetro homogêneo e espaços interfibrilares ampliados (Hukins & Aspden, 1985; Kühn, 1987; Maroudas, 1990).

Estudos com degradações enzimáticas dos componentes proteoglicânicos demonstraram que a forma da cartilagem é extremamente dependente da integridade da malha colagênica (Harris *et al*, 1972). Mais recentemente, tem-se atribuído crescente importância à malha colagênica nas propriedades de tensão e de resistência à compressão exibidas pelas cartilagens (Broom, 1984; Schmidt *et al*, 1990), o que certamente decorre do tipo de malha tridimensional e com ligações cruzadas estabelecida pelas fibrilas. Enquanto o colágeno responde pela resistência à deformação, a presença dos proteoglicanos parece atuar reduzindo a velocidade com que a matriz se deforma (Schmidt *et al*, 1990). Sem os proteoglicanos, a malha colagênica apresenta propriedades limitadas. Assim, a resistência à tensão imposta pelo colágeno é atingida rapidamente na ausência dos proteoglicanos (Schmidt *et al*, 1990) (o que significa grande deformação frente a pequenas tensões). Por outro lado, uma vez depletada dos proteoglicanos, a matriz cartilaginosa colapsa frente a forças de compressão, sem a capacidade apresentada pela cartilagem articular intacta de difundir a carga imposta (Broom & Poole, 1983).

Alguns dos tratamentos enzimáticos foram capazes de reproduzir os aspectos apresentados pela cartilagem osteoartrósica, no que se refere à tendência de agregação lateral das fibrilas de colágeno, frequentemente formando fibras, obviamente sem capacidade de resistir a forças de compressão (Broom, 1988).

Já com respeito aos proteoglicanos, existe na cartilagem articular um predomínio de moléculas de agrecam, o grande proteoglicano contendo condroitim sulfato e queratam

sulfato, com capacidade de interação com o ácido hialurônico e de formar grandes agregados com massa molecular total ao redor de 10^7Da (Heinegård & Axelsson, 1977; Heinegård & Sommarin, 1987a; Hascall & Hascall, 1983; Hascall, 1988). O agrecam apresenta grande complexidade estrutural, sendo formado por um *core* protéico que apresenta três domínios globulares (G1 e G2, na porção N-terminal, e G3, na porção C-terminal) e pelo menos duas regiões lineares (o domínio interglobular (IGD), entre G1 e G2, e o domínio onde se ligam até cem cadeias de condroitim sulfato e cinquenta cadeias de queratam sulfato, entre G2 e G3) (Poole, 1986; Paulsson *et al*, 1987; Hascall 1988; Antonsson *et al*, 1989; Kjellén & Lindahl, 1991; Iozzo, 1998). Enquanto as cadeias de condroitim sulfato são longas, com massa molecular entre 15.000 e 40.000 Da, as cadeias de queratam sulfato são curtas, com massa molecular entre 5.000 e 8.000 Da.

As cadeias de condroitim sulfato ocupam dois domínios adjacentes no *core* protéico (CS1 e CS2), que diferem entre si pela densidade dos sítios de glicosilação. A cadeia de condrotim sulfato pode ser divida em três diferentes regiões: a região oligossacarídica de ligação ao *core* protéico (*ser*-xyl-gal-gal-glcA-) (Sugahara *et al*, 1988; Shibata *et al*, 1992; Chen *et al*, 1996), a região de dissacarídeos repetitivos (-galNAcS-glcA-) (Weitzhandler *et al*, 1988; Deutsch *et al*, 1995; Bayliss *et al*, 1995; Roughley *et al*, 1996) e a região que possui o resíduo do terminal não redutor (-glcA ou galNAcS) (Otsu *et al*, 1985; Midura *et al*, 1994; Midura *et al*, 1995; Plaas *et al*, 1996; Plaas *et al*, 1997; Plaas *et al*, 1998). Uma heterogeneidade na sulfatação dos resíduos das hexoses e hexosaminas têm sido encontrada em todas as regiões, podendo assim influenciar as propriedades físico-químicas das cadeias de glicosaminoglicanos. Esta heterogeneidade pode fornecer um mecanismo pelo qual a matriz cartilaginosa pode se adaptar às diversas situações de uma articulação.

As cadeias de queratam sulfato são constituídas por dissacarídeos repetitivos (Gal-GlcNAc). Estes dissacarídeos podem ser monossulfatados ou dissulfatados, sendo que esta sulfatação pode ocorrer no carbono 6 dos resíduos de galactose e/ou de N-acetilglicosamina (Bhavanandan & Meyer, 1968). Os tipos de queratam sulfato são classificados de acordo com sua ligação ao core protéico (Meyer, 1970). O queratam sulfato presente na molécula de agrecam é chamado de KS-II por estar ligado através de uma N-acetilgalactosamina a uma serina ou treonina. Este tipo de queratam sulfato possui ainda uma subclassificação. É chamado de KS-II-A Articular por conter resíduos de fucose e ácido N-acetilneuramínico

ligados à sua cadeia (Nieduszynski *et al*, 1990). Este glicosaminoglicano está presente em todas as moléculas de agrecam já caracterizadas, exceto em agrecans de condrossarcoma de ratos (Choi *et al*, 1971; Oegema *et al*, 1975).

Além dos glicosaminoglicanos, o agregam possui ainda cerca de 60-75 oligossacarídeos (O- e N-ligados) (Lohmander *et al*, 1981; Wight *et al*, 1991).

A interação com o ácido hialurônico dá-se através do domínio G1 e é reforçada pela associação de glicoproteínas especiais denominadas proteínas de ligação ou "link". Para atingir o enorme tamanho mencionado acima, várias moléculas do agrecam se associam a uma mesma molécula de ácido hialurônico. Aparentemente, este agregado é grande o suficiente para que seja retido na matriz cartilaginosa pela malha colagênica, aparentemente por simples restrição física, uma vez que o monômero livre difunde-se livremente da carilagem (Carney & Muir, 1988).

A grande densidade de cargas negativas apresentadas pelos proteoglicanos e, consequentemente, pelos agregados resultam numa grande retenção de água nos tecidos onde são encontrados (Carney & Muir, 1988).

Nas cartilagens especificamente, o volume atingido pelos agregados é restringido pela malha colagênica a cerca de 20% de máximo que poderia alcançar (Hascall, 1988). Desta forma, cria-se no tecido uma grande turgescência, que é em grande parte responsável pela capacidade de resistência à compressão apresentada pelas cartilagens. Nas osteoartrites, por exemplo, existem modificações da malha colagênica, que se torna incapaz de restringir o volume ocupado pelos proteoglicanos, resultando na incapacidade de resistir às forças de compressão e na degeneração da cartilagem (Maroudas, 1976).

As relações quantitativas entre colágeno e proteoglicanos variam entre diferentes cartilagens e entre diferentes regiões de uma mesma cartilagem (Harab & Mourão, 1988; Mourão, 1988; Heise & Toledo, 1993), assim como varia conforme o desenvolvimento e o envelhecimento (Bayliss, 1990) e nos processos degenerativos (Michelacci *et al*, 1979; Lohmander, 1994).

Apesar disto, a presença de proteoglicanos em grande quantidade em associação com o colágeno não implica necessariamente no mesmo arranjo observado nas cartilagens e nas propriedades por elas apresentadas. São exemplos desta restrição, as regiões

fibrocartilaginosas dos tendões sujeitos a forças de compressão e, talvez, as fibrocartilagens em geral (Carvalho, 1995).

Embora diferentes mamíferos tenham servido de modelos experimentais para estudos sobre a cartilagem articular, o que resultou em enorme conhecimento sobre estes tecidos, pouco se conhece sobre as cartilagens articulares de animais de grupos inferiores, sobre a sua estrutura e composição, e mesmo sobre o comportamento apresentado durante o desenvolvimento e/ou envelhecimento. Menos ainda se conhece sobre alterações patológicas.

Estendendo nossos estudos sobre a matriz extracelular de anuros, que até agora se concentraram em um tendão sujeito a forças de compressão de rãs (Carvalho & Vidal, 1994a, 1994b, 1994c), estamos propondo o estudo das cartilagens articulares do joelho de *Rana catesbeiana*.

O interesse sobre este material reside em dois fatores principais. Primeiro, já foi demonstrado que estes animais não possuem crescimento endocondral, tendo sido sugerido que o crescimento dos ossos longos ocorra através do periósteo (Kemp & Hoyt, 1969). Segundo, em trabalho anterior, verificamos a existência de regiões de proliferação, de hipertrofia e, aparentemente, de degeneração, sem a ocorrência de ossificação endocondral (Felisbino & Carvalho, 1999). Terceiro, parece que o crescimento ósseo está acoplado a um sistema de degradação da cartilagem que se estende pela matriz cartilaginosa em diferentes extensões.

Parece então plausível verificar se os mesmos mecanismos envolvidos com a estabilização da estrutura e com a fisiologia das cartilagens articulares e de crescimento de mamíferos estariam presentes em outros grupos, em especial nos anfíbios, que ocupam uma posição inferior na escala evolutiva.

Neste sentido, como parte de um projeto mais amplo que visa investigar estrutural e bioquimicamente alguns dos principais componentes macromoleculares da cartilagem epifisária de rãs, seu desenvolvimento e fisiologia na resistência à compressão e no crescimento dos ossos, procurando indentificar características comuns com as cartilagens de mamíferos e aspectos que lhe sejam peculiares, este trabalho esteve voltado à caracterização do grande proteoglicano presente na cartilagem aritcular de rãs, com respeito à microestrutura do core protéico e dos glicosaminoglicanos e capacidade de formar agregados com o ácido

hialurônico. Além disto serão investigadas variações associadas à regiões da cartilagem epifisária distintas anatômica e histologicamente.

IV - Objetivos Gerais

Caracterizar o grande proteoglicano da cartilagem epifisária de *Rana catesbeiana* (Rãs-touro), suas características estruturais, características dos glicosaminoglicanos e a capacidade de agregação com ácido hialurônico, considerando regiões específicas identificadas morfologicamente e verificando a conservação filogenética de sua estrutura.

V - Artigos

1 – Calcification in the anuran cartilage: mapping, identification of ectopic site and some associated biochemical aspects.

Será submetido ao Tissue and Cell.

2 – Aggrecan structure in the amphibian cartilage.

Submetido ao Brazilian Journal of Medical and Biological Research.

3 – Amphibian aggrecan: Core protein analyses and regional aspects of glycosaminoglycan substitution in the bullfrog.

Será submetido ao Matrix Biology.

Calcification in the anuran cartilage: mapping, identification of ectopic site and some associated biochemical aspects

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Abstract. The epiphyseal cartilage (EC) of the bullfrog shows distinctive aspects as compared to mammalian and avian models. The growth cartilage is not involved with in longitudinal growth of long bones, which depends mostly on periosteal ossification. In this paper we have checked whether the growth cartilage, though not involved in trabecular bone formation, undergoes calcification. Besides sites of calcification in the hypertrophic cartilage, we also found ectopic sites of calcification in other regions of the EC. Histochemistry revealed a condensation of proteins and proteoglycans at the sites of calcification and showed alkaline phosphatase activity on the surface of chondrocytes in the calcification areas. In addition, we have followed the formation of calcium crystals at the TEM level. The calcification sites in the growth cartilage start as small dots which grow up and fuse together, while calcification in ectopic regions showed no distinct morphological pattern and leads to disruption of the organic matrix. Biochemically, the growth cartilage differs from other EC regions by showing increased hydration and also a higher hydroxyproline/uronic acid ratio as well as slightly longer chondroitin sulfate chains. We also discuss the possibility that ectopic calcification results from the diffusion of calcification signals from the periosteum or from localized high pressure zones in the lateral cartilage.

Introduction

Cartilage matrix calcification preceeds bone formation in the endochondral ossification which is a series of events responsible for the longitudinal growth of long bones in mammals and birds (Breur *et al.* 1991; Cancceda *et al.* 1995; Erlenbacher *et al.* 1995, Bianco *et al.* 1998). Calcification of the hypertrophic cartilage is a coordinated process involving the hypertrophic chondrocytes in the growth plate. These cells produce type X collagen (Schmid and Conrad 1982; Schmid and Linsenmayer 1983; Gibson and Flint 1985; Kwan *et al.* 1991; Sandell et al. 1994) and matrix vesicles (Leboy *et al* 1989; Anderson 1995; Kirsch *et al.* 1997) and show alkaline phosphatase activity (Poole 1991; Roach 1991), which is thought to be directly or indirectly involved with calcium crystal formation. Some authors believe that calcification leads to chondrocyte apoptosis (Hatori et al. 1995; Kim 1995; Zenmio *et al.* 1996; Gibson *et al.* 1997; Hashimoto *et al.* 1998).

Previously we have shown that besides structural distinctions as compared to the mammalian and avian epiphyseal cartilages, the epiphyseal cartilage of the bullfrog is not involved with endochondral bone formation and is associated with the radial expansion of the bone (Felisbino & Carvalho, 1999). Though not involved with endochondral bone formation, the epiphyseal cartilage showed sites of calcification in the growth cartilage and ectopic sites of calcification in the articular cartilage. In this paper, we have mapped the calcification sites in the epiphyseal cartilage of the bullfrog, defined some of them as ectopic, characterized the pattern of crystal growth and the associated organic matrix modifications and investigated some biochemical characteristics related to the glycosaminoglycans present in each region. We concluded that ectopic calcification takes place as a result of the action of chondrocytes responding to unrestricted signals difusing from the periosteum.

Material and methods

Animals: Specimens of *R. catesbeiana* were obtained from a farm in Atibaia (São Paulo State, Brazil). Animals were considered reproductively mature, by examination of the gonads. They were killed by decaptation after cold immobilization and the distal femoral epiphyseal cartilage was dissected out and immediately fixed by immersion in different fixatives as described below or frozen at –70°C for the biochemical analyses.

Histochemisty: Cartilage fragments were fixed in 4% formaldehyde in phosphate buffer at pH7.4, containing 0.85% NaCl (PBS) for 24hours. Some samples were decalcified for 15 days in a solution containing 4% formaldehyde, 10% acetic acid and 0.85% NaCl. The material was then dehydrated in a graded ethanol series, clarified in Cedar wood oil and embedded in Paraplast Plus embedding medium. Serial 6μm-thick sagital sections were stained with toluidine blue, picrosirius-hematoxylin (PSH) or subjected to the von Kossas reaction for calcium. Some sections were examined unstained with the phase contrast microscope and those stained with picrosirius were also examined by polarization microscopy.

Enzyme histochemistry: Some tissue fragments were fixed in 4% formaldehyde in 0.1M cacodylate buffer at pH 7.2 for 4 hours, washed with the same buffer, treated with 70% ethanol and embedded in JB4 historesin. Two micrometers thick sections were assayed for alkaline phosphatase (AlkPase) activity using 0.1% α-naphtyl phosphate, 0.1% fast red and 50mM $MgCl_2$ in 0.1M tris-maleate buffer at pH10. The controls were prepared using the same solutions without α-naphtyl phosphate (Bancroft 1982; Cole and Wezeman 1987). Sections were counter-stained with methyl green.

Transmission electron microscopy: Selected areas of the epiphyseal cartilage were processed for transmission electron microscopy. Fragments ($1mm^3$) were fixed with 3% glutaraldehyde plus 0.25% tannic acid and 5.4% sucrose in Millonig's buffer (Cotta-Pereira

et al. 1976). Some fragments were decalcified with EDTA. Tannic acid was chosen for a better preservation and visualization of fibrillar components in the cartilage extracellular matrix (Eggli *et al.* 1985). After washing with the same buffer the material was post-fixed with 1% osmium tetroxide, washed again, dehydrated in graded acetone, and then embedded in Epon. Sections (1μm) were cut and stained with toluidine blue for light microscopy. Ultrathin sections (silver-gold interference colors) were cut on a LKB ultramicrotome using diamond knivesand contrasted with alcoholic uranyl acetate and lead citrate. The grids were examined in a Phillips 301 transmission electron microscope operating at 80 kV.

Biochemical Analyses

Water content: Water content was calculated by weighing samples before and after treatment with acetone and vacuum drying to constant weight.

Sulfated glycosaminoglycans and uronic acid quantfication: Fifty miligrams of each articular, lateral and growth cartilages were digested with papain (Merck) (Harab & Mourão, 1989). The insoluble fraction was removed by centrifugation. The soluble fraction was analysed for their content in sulfated glycosaminoglycans (sulfGAG) by the dimethylmethylene blue method (DMMB) (Farndale *et al*, 1986) and uronic acid by the orcinol reaction (Brown, 1946). Mixed chondroitin sulfate isomers (Whale cartilage, Sigma) were employed as standard.

Hydroxyproline quantification: Articular, lateral and growth cartilage (50mg) were dryed as described above and hydrolysed in 6M HCl at 130°C for 4h. The hydroxyproline content was determined colorimetrically (Stegeman & Stalder, 1967) using hydroxyproline (Merck) as standard.

Sephadex G-200 Chromatography: Glycosaminoglycans from each cartilage (280μg) were subjected to gel filtration on a Sephadex G-200 column (Pharmacia) (1.2x25cm)

equilibrated and eluted with destilled water. The flow rate was 2.4ml\h. The fractions (630µl) obtained were assayed for their content in sulfated GAG by the DMMB assay.

Results

Structural and ultrastructural aspects

Calcium deposits were identified by the von Kossa's procedure. They were dispersed in the growth cartilage and appeared as isolated or coalesced globular structures with no clear distribution pattern (Fig. 1). Calcium was also localized in some areas of the lateral cartilage (at a uniform distance from the periosteal bone), but in this region the calcium deposits were not globular as in the hypertrophic cartilage, but showed an irregular profile (Fig. 2 and 3). Fig. 3 depicts the distribution of calcium deposits in the epiphyseal cartilage of the bullfrog, considering its major structural elements. Using phase contrast microscopy the calcification sites in decalcified material could be visualized. They corresponded to disrupted areas in the lateral cartilage, close to the periosteal bone tip (Fig. 5). In the hypertrophic cartilage, the spherial structures were still identified after decalcification, showing an increased concentration of the organic matrix towards the center, and readily seen limits from the non-calcified areas (Figs. 6 and 7).

After staining with picrosirius the calcification sites showed a more intense staining in both the lateral cartilage (Fig. 8) and in the hypertrophic cartilage (Fig. 11). In the lateral cartilage the calcification sites corresponded to areas of disrupted cartilage matrix (Fig. 9). Analyses of the picrosirius stained sections using the polarizing microscope revealed increased birefringence of the cartilage matrix close to the calcification site, representing a tighter packing of the fibrillar components (Fig. 10). The spherical structures corresponding to the calcium deposits were resistant to decalcification and showed a gradient of staining, deeper towards the center of the structures. The PSH staining clearly revealed that these spherical structures fused together as they grew up.

Fig. 12 shows the toluidine blue staining of the calcification sites of the lateral cartilage. Decalcification leads to disruption of the organic matrix, which showed a banded organization of deep- and faint-stained matrix.

Chondrocytes of the lateral cartilage and in the growth cartilage associated with the calcification sites showed a positive reaction after the cytochemical procedure for the identification of alkPase activity (Fig. 13). The reaction product was concentrated at the plasma membrane.

At the electron microscopy level, calcium accumulation was observed as different structures. Firstly it was detected in association with membrane-bound round structures (Fig. 14). Secondly, it was detected in the collagen fibrils of the condensed cartilage matrix (Fig. 15). Thirdly, it appeared as dense granules amongst the fibrillar components in the condensed cartilage matrix (Fig. 16) and, finally, it appeared as highly mineralized, rounded structures,which apparently grow by the deposition of calcium in the adjacent collagen fibrils and non-fibrillar matrix (Fig. 17).

In the decalcified material, we could observe the integrity of the chondrocytes in close association with the condensed cartilage matrix corresponding to the calcification sites (Fig. 18). The spherical structures of the growth cartilage were observed as areas of condensation of fibrillar and non-fibrillar matrix components (Fig. 19). The higher density of the matrix in these spherical structures is clearly seen in Fig. 20. Fig. 20 also shows the presence of a disorganized material in between the coalesced spherical structures, which may well be cellular residues. In the lateral cartilage, matrix disruption and the banded aspects observed at the light microscopy level could also be seen (Figs. 21 and 22). The extracellular matrix is reorganized in bands composed of fibro-granular material with no visible collagen fibrils.

Compositional aspects

Table I summarizes the biochemical results obtained herein. The growth cartilage showed higher hydration, possessing up to 80% water, as compared to the 74% and 65% values exhibited by the articular and lateral cartilage, respectively. In contrast, the amount of hydroxyproline was lower (Table I). The growth cartilage also showed an increased content in sulfated GAG and uronic acid (Table I and Fig. 23). The uronic acid to sulfated GAG and the uronic acid to hydroxyproline ratios were higher in the growth cartilage as compared to the other two regions (Fig. 24).

The measurement of the hydrodynamic size of the chondroitin sulfate chains by chromatography on Sephadex G200 (Fig. 25), showed that those present in the growth cartilage were slightly longer. The chondroitin sulfate chains in the articular cartilage were highly polydisperse and contained the shorter chains.

Discussion

The epiphyseal cartilage of the bullfrog shows structural adaptations differing from the mammalian and avian counterparts in many aspects (Felisbino and Carvalho, 1999) but resembling other anuran species (Dickson 1982; DellOrbo et al. 1992). In this paper we have identified and mapped calcification sites in each of the three main regions of this cartilage. Calcium deposits were seen in the articular, lateral and growth cartilage, concentrating in the two latter ones. Calcium deposits were uniformily rounded in the hypertrophic cartilage but not in the other regions. The rounded structures of the hypertrophic cartilage grew up and fused together and showed no clear distribution pattern, as observed in mammals, where calcification occurs in the longitudinal septa (Hunziker and Schenk 1989; Gentili *et al.* 1993; Hunziker 1994). The rounded structures showed a denser core and a clear limit from the adjacent cartilage matrix. Apparently they arise from matrix vesicles produced by the chondrocytes, which also exhibited alkaline phosphate activity. The membrane bounded round structures observed by TEM are probably matrix vesicles.

The calcification of the growth cartilage is not necessarily associated with the formation of bone trabeculae, which may be formed in contact with non-calcified cartilage and appear much later in these animals (Felisbino and Carvalho, in preparation).

We consider that the calcification sites found in the lateral cartilage and those close to the proliferating zones of the growth cartilage are ectopic. In the lateral cartilage it is very clear that calcification leads to the disorganization of the cartilage matrix, and may lead to a general disfunction of the cartilage. Though ectopic, these calcification sites seem to arise by the action of the adjacent chondrocytes, which were normal at the ultrastructural level and

showed intense alkaline phosphatase activity. So, these ectopic sites of calcification could not be related to a reaction following (traumatic) necrosis of the cartilage cells.

The aspects of calcium deposition seemed normal, as compared to the mineralization aspects of the classical models of cartilage calcification. However, the different growth pattern observed between the structures of calcium deposits in the growth cartilage and those on the lateral cartilage, as well as the aspects of the organic matrix after decalcification, may reflect differences in the control of calcium deposition.

The biochemical analyses revealed differences in the content of collagen (as measured by the content of hydroxyproline) and proteoglycans (as measured by the content in sulfated GAGs and uronic acid), as well as in the length of the chondroitin sulfate chains. Current studies in the lab aim at further increasing our knowledge on the fine structure of the chondroitin sulfate chains and the aggrecan, and their variations according to the different regions of the epiphyseal cartilage, aiming at establishing correlations with the calcification aspects described in this paper.

Based on the structural aspects of the calcium deposits in the different regions of the epiphyseal cartilage, and the apparent involvement of the chondrocytes in producing them, we may suggest that the ectopic sites of calcification arise from unrestricted signalling emanating from cells associated with the growth of the periosteal bone tip in this animal. Since the ectopic calcification of the lateral cartilage appears at a constant distance from the periosteal bone, their origin may be attributed to either a diffusible factor emmanating from the periosteal bone (where calcification/ossification occurs) or from localized high pressure exerted by the periosteal bone during joint loading.

We are currently investigating the consequences of the existence of these ectopic sites of calcification on the joint physiology of these anurans and also the expression of calficication associated components of the cartilage matrix, such as type X collagen.

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Figure legends

Figs. 1-3. von Kossa's staining of the bullfrog epiphyseal cartilage. Positive reaction was found in spherical structures of the growth cartilage (Fig. 1) and in the lateral and upper proliferating zones, close to the periosteal bone (pb)(Fig. 2). Fig. 3 is a detail of the calcification sites in the lateral cartilage, showing their irregular profile. Figs. 1 and 2, X250; Fig. 3, X625.

Fig. 4. Schematic drawing of the epiphyseal cartilage of the bullfrog, showing the distribution of calcium deposits as revealed by the von Kossa's procedure, with respect to the major structures. The asterisk marks a cartilage canal.

Figs. 5-7. Phase contrast microscopy of unstained sections of the bullfrog epiphyseal cartilage. Fig. 5 corresponds to the tip of the periosteal bone (arrow), showing cells that apparently erode the cartilage as the bone tip grows. The asterisk marks a disrupted area of the lateral cartilage, corresponding to an ectopic site of calcification after decalcification. V = blood vessel. Fig. 6 corresponds to the transition between the hypertrophic cartilage and the bone marrow (bm). Fig. 7 corresponds to another area of the hypertrophic cartilage to marrow transition, showing coaslesced spherical structures and the dense nuclei from where they originated (arrowheads). The arrows point to mononucleated cells that are associated with a cartilage canal under formation. Figs. 5 and 6, X 250; Fig. 7, X625.

Figs. 8-11. PSH stained sections of the bullfrog epiphyseal cartilage. Fig. 8 shows the lateral cartilage (lc) and the upper proliferating zone (upz) of the growth cartilage, close to the periosteal bone (pb) and the osteochondral ligament (ocl). The arrows point to the calcification sites, which stained deeper than the adjacent cartilage matrix. In the lateral cartilage, they form a continuous line at a given distance from the ocl. Fig. 9 is a details of a calcification site in the lateral cartilage, showing that the stained structures are irregularly distributed, and show a higher packing of fibrillar components, as seen under polarized light (Fig. 10). Fig. 11 is an aspect of the spherical structures observed in the hypertrophic

cartilage. The spherical structures showed marked limits with the adjacent matrix and a gradient of staining, deeper to the center. Some of them fused to each other. Fig. 8, X250; Figs 9 and 10, X 430; Fig. 11, X1075.

Fig. 12. Toluidine blue staining of the lateral cartilage of the bullfrog, showing aspects associated with the calcification sites. As a consequence of decalcification, the matrix is disrupted and shows discontinuities (stars). The organic matrix shows a banded pattern with alternating deep- and faint-stained strips (arrows). X430.

Fig. 13. Cytochemical detection of the alkPase activity. The chondrocytes at the calcification sites in the lateral cartilage show conspicuous alkPase activity (arrows), which concentrate at the cell surface. The chondrocytes in the upper proliferating zone (upz) also depicted alkPase activity. The osteoblasts (ob) close to the periosteal bone (pb) also showed activity. X430.

Fig. 14-17. Transmission electron microscopy of the non-decalcified epiphyseal cartilage of the bullfrog. Fig. 14 shows a membrane bound (arrows) structure with apparent deposition of calcium, amongst the collagen fibrils. X110,000. Fig. 15 shows a calcification site in the lateral cartilage, showing a well defined limit to the adjacent matrix (arrowheads) and collagen fibrils which show deposits of calcium (arrows). The asterisk marks a zone where calcium crystal deposition has extended to the non-fibrillar matrix. X17,250. Fig. 16 shows two adjacent sites of calcification (asterisks), which show a denser center and some globular electron-dense structures. The arrows show collagen fibrils in the adjacent matrix which were mineralized. X57,350. Fig. 17 corresponds to a site of calcification in which calcium deposition is more advanced. The structure is completely occupied by mineral and the adjacent matrix also shows signs of calcium deposition, as irregular structures. X40,250.

Figs. 18-22. Transmission electron microscopy of the decalcified cartilage of the bullfrog. Fig. 18 shows a chondrocyte close to a calcification site (asterisk). The cell has an intact plasma membrane and typical pore complexes and ribosomes. X 110,000. Fig. 19

corresponds to a spherical structure from the hypertrophic cartilage showing a denser organic matrix. X9,800. Fig. 20 is an aspect of coalesced spherical structure. There are some unidentified structures in between the former spherical structures, which apparently correspond to cellular residues. X6,000. Fig. 21 is a low power view of the organic matrix remaining in the calcification sites after decalcification. The banded pattern observed by light microscopy is recognized. Decalcification resulted in disruption of the matrix. X4,890. Fig. 22 is a detail showing the fibro-granular nature of the alternating clear and dark bands. X33,600.

Fig. 23. Content of sulfated GAGs, uronic acid and hydroxyproline in the different regions of the bullfrog epiphyseal cartilage. AC = articular cartilage, LC = lateral cartilage, GC = growth cartilage. Values are in micrograms per mg of wet tissue.

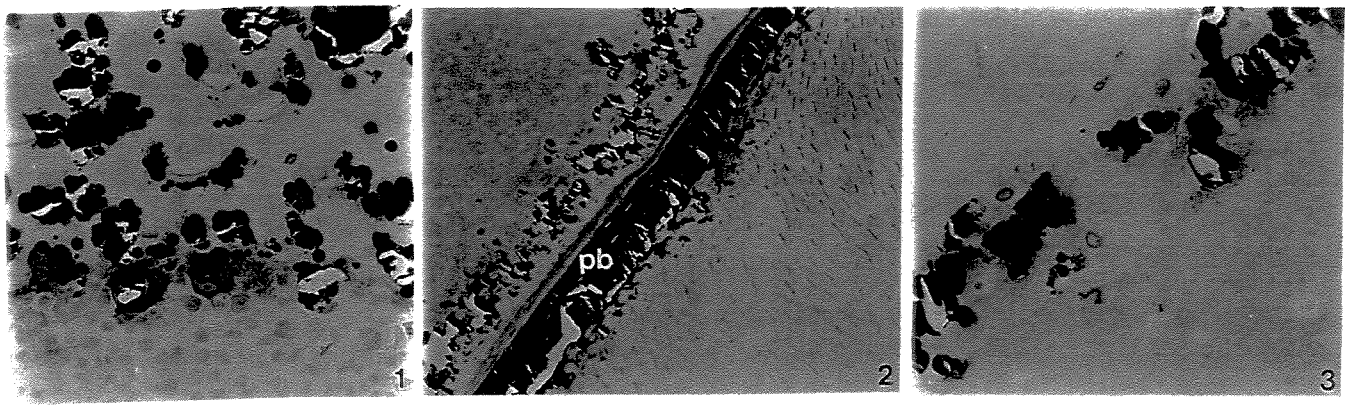
Fig. 24. Ratios of uronic acid to hydroxyproline and uronic acid to sulfate GAG in the different regions of the epiphyseal cartilage. Values were calculated according to the values presented in Table I. AC = articular cartilage, LC = lateral cartilage, GC = growth cartilage.

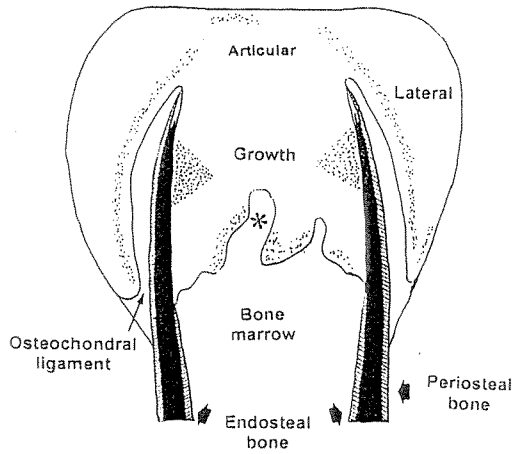
Fig. 25. Hydrodynamic size of chondroitin sulfate chains present in the different regions of the epiphyseal cartilage. Fractions from the chromatography on Sephadex G200 were analysed for the content of sulfated GAGs, using the DMMB procedure. Chondroitin sulfate chains from the growth cartilage are slightly longer that those from the other two regions. The chondroitin sulfate chains from the articular cartilage showed the more polydisperse behavior and contained the shorter structures.

Table I. Biochemical analyses of the different regions of the bullfrog epiphyseal cartilage regions

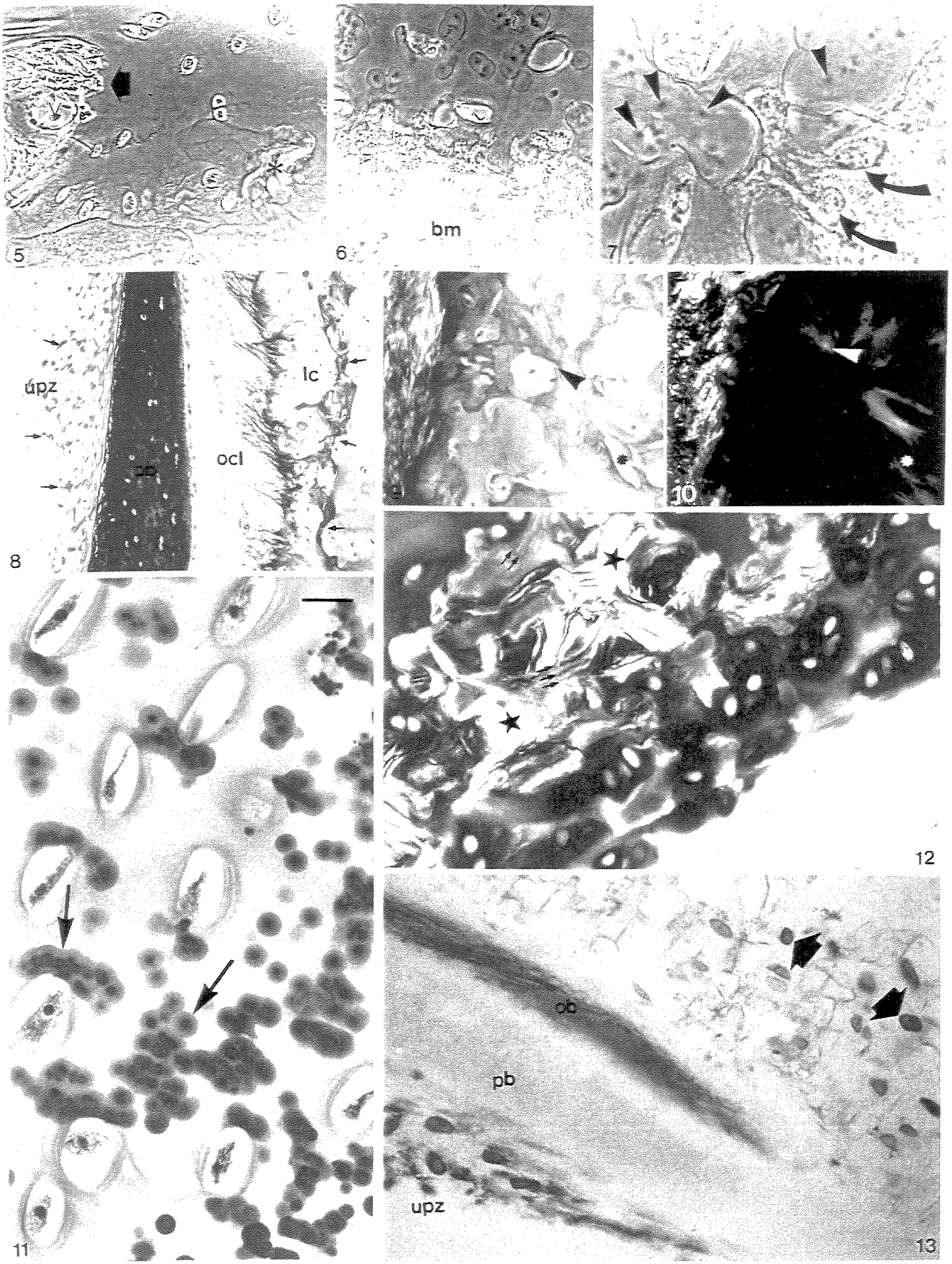
Cartilage region	Water content	HO-proline[1] (μg/mg of wet tissue)	Uronic Acid[2] (μg/mg of wet tissue)	Sulfated GAG[3] (μg/mg of wet tissue)
Articular	74%	53 ± 0.1	323 ± 24	288 ± 28
Lateral	65%	42 ± 2	358 ± 100	257 ± 21
Growth	80%	34 ± 2	545 ± 53	325 ± 66

[1]Determined by the procedure of Stegemann and Stalder (1967)
[2]Determined by the orcinol reaction (Dische, 1946)
[3]Determined by the DMMB procedure (Farndale et al. 1986)





Areas of calcification Bone matrix Areas of ossification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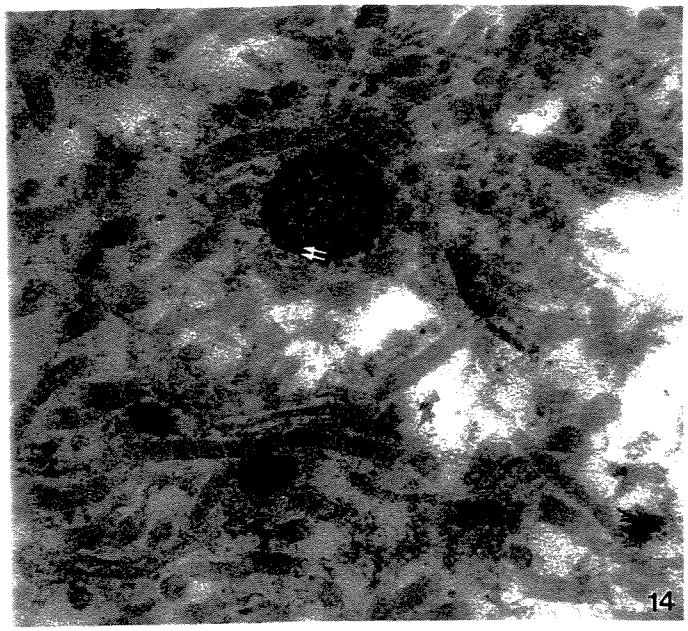

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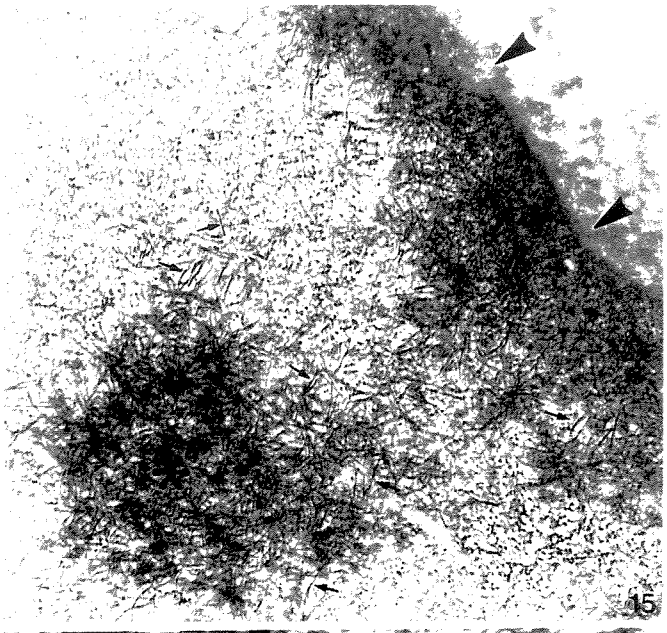

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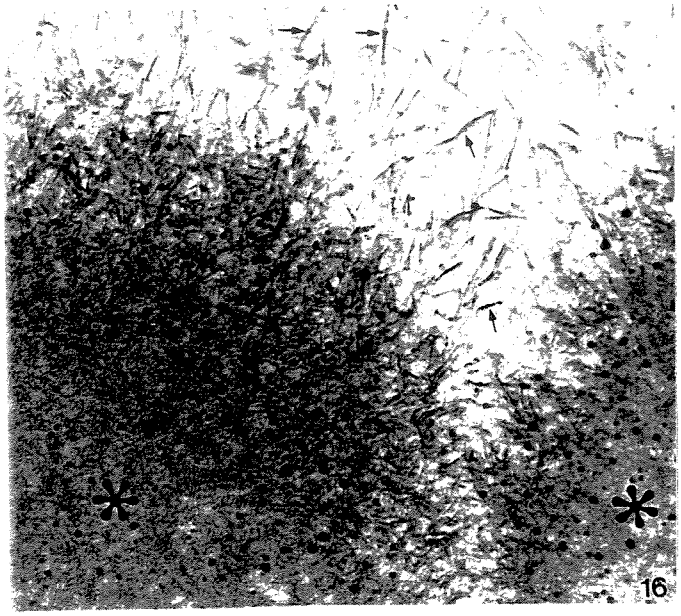

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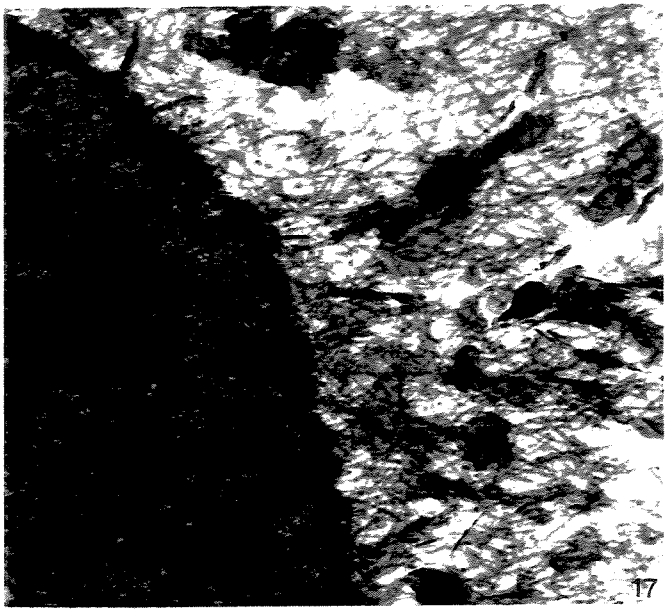

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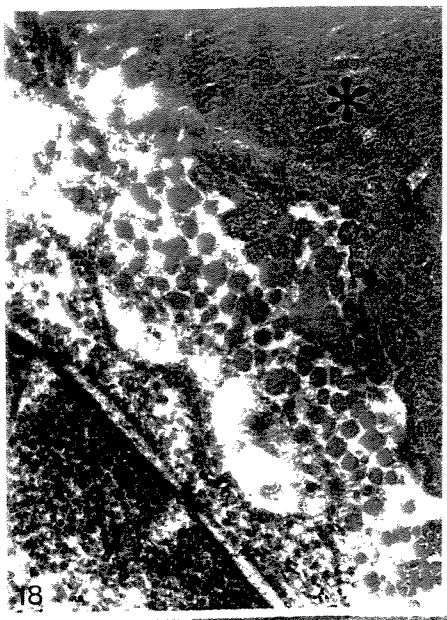

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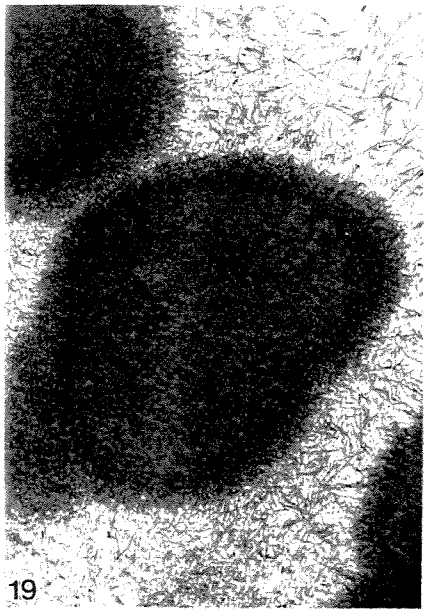

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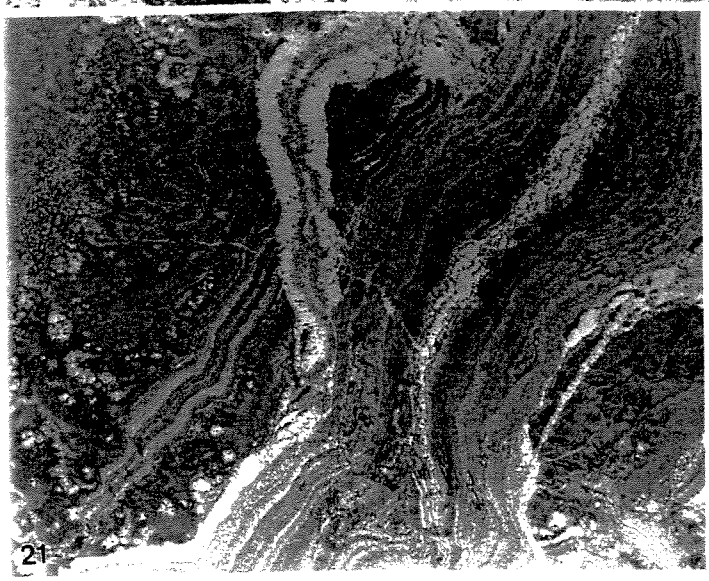

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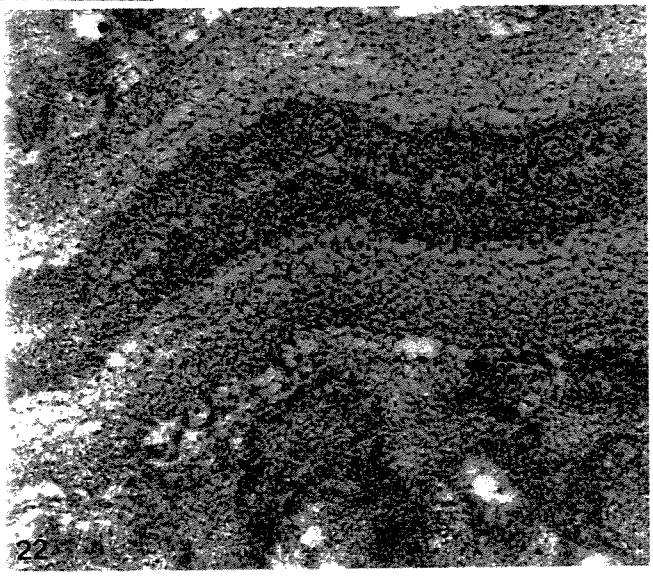

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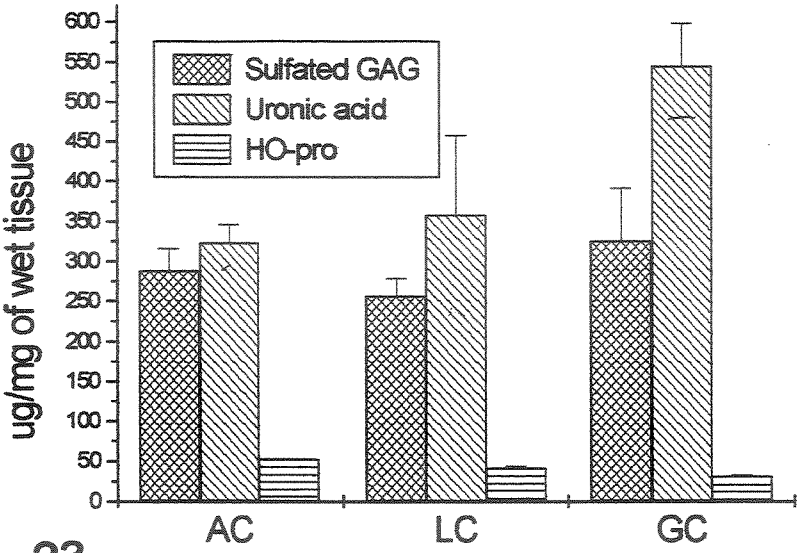
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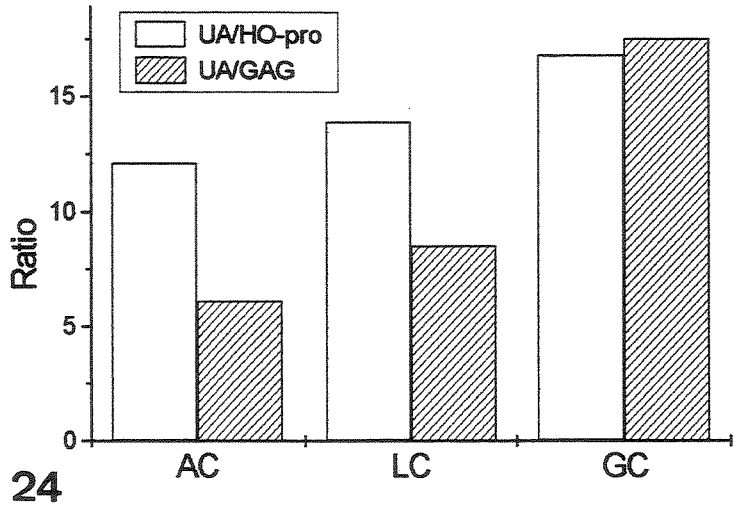
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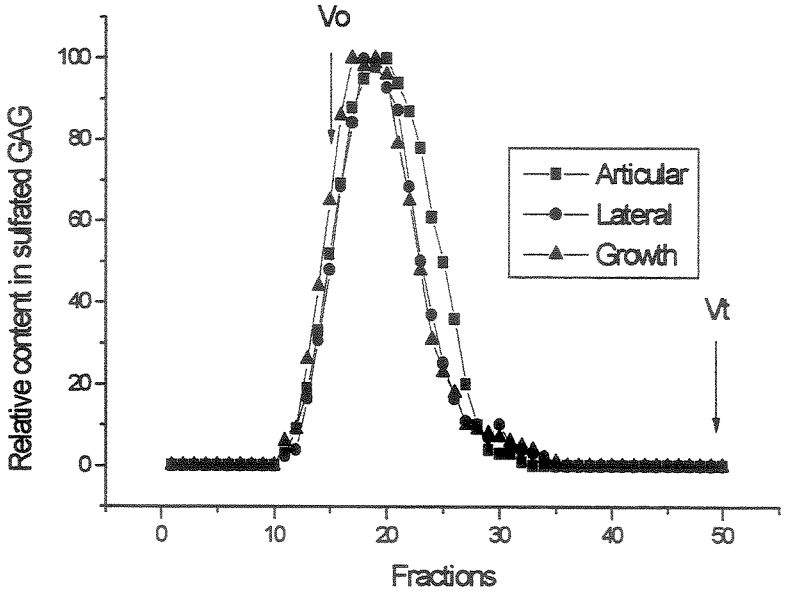
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Aggrecan structure in the amphibian cartilage

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Running title: Structure of the bullfrog aggrecan

Key words: aggrecan, bullfrog, cartilage, chondroitin sulfate, epiphyseal cartilage

Abstract

The structure of the large proteoglycan present in the bullfrog articular cartilage was studied by immunochemical and biochemical essays. The isolated monomer showed a polydisperse behavior on Sepharose CL2B, with a peak at Kav= 0,14. The chondroitin sulfate (CS) chains showed a Kav of 0.4 after chromatography on Superose 6 and a ΔDi4S/ΔDi6S ratio of 1.6 as determined by HPLC on a AS4A column. This methodology allowed for the identification of GalNAc4S and GalNAc4,6S as the main non-reducing terminals of the CS chains and the estimation of an average chain length of 38 disaccharides. Keratan sulfate (KS) was identified by the use of two monoclonal antibodies on Western blots after chondroitinase ABC treatment. A KS-rich region (~110kDa) was isolated by a sequential treatment with chondroitinase ABC and proteases. We also employed antibodies in Western blotting experiments and showed that the full length deglycosylated core protein is about 300kDa after SDS-PAGE. Domain specific antibodies revealed the presence of immunoreactive sites corresponding to G1/G2 and G3 globular domains and the characterization of this large proteoglycan as aggrecan. The results reveal a high conservation of the aggrecan domain structure in this lower vertebrate.

Introduction

Cartilage is a resilient tissue able to resist tension and pressure forces (1). These properties are thought to result from a highly coordinated array of various components, amongst which are type II collagen, large and small proteoglycans, and other non-collagenous glycoproteins (2,3). The large proteoglycan aggrecan consists of a core protein to which the glycosaminoglycans (GAGs) chondroitin sulfate (CS) and keratan sulfate (KS), and N- and O-linked oligosaccharides are attached (4). The GAGs have a high negative fixed-charge density, conferring on aggrecan its characteristic osmotic activity (5) which, in addition to its capacity to aggregate with hyaluronan and participate in a concerted interplay with type II collagen fibrils, endows the tissue with the ability to withstand compressive forces and to distribute the load (6).

The aggrecan core protein has different domains. The G1 domain at the N-terminal consists of three subdomains and binds to hyaluronan and link protein in huge aggregate structures (7-9). The interglobular domain (IGD), located between the G1 and G2 domains, is a proteolytically sensitive region of the molecule, centrally involved in aggrecan catabolism (10-14). The G2 domain (15) consists of two subdomains also present in G1. The function of G2 is still unclear. The two GAG substitution domains correspond to a KS-rich region with about 50 chains (16), and a CS-rich region with about 100 chains (17). The G3 domain consists of EGF-like, lectin-like (18) and CRP-like motifs, which also occur together in some cell adhesion molecules (19).

We have studied the bullfrog epiphyseal cartilage and reported differences in structure, as well as in its involvement with long bone growth (20). This led us to believe that the identification and characterization of the macromolecules in the bullfrog cartilage would be important for the understanding of its physiology at the cellular and molecular levels.

Since aggrecan function is essential to the physiology and structure of cartilage, we used immunochemical tests to elucidate some of the characteristics of the core protein and biochemical assays to determine some aspects of the attached GAGs. Considering that amphibians are lower vertebrates, the results presented here demonstrate a high conservation of the domain structure of aggrecan.

Material and Methods

Preparation of the large proteoglycan from the bullfrog epiphyseal femoral distal cartilage

One-year-old bullfrogs, *Rana catesbeiana,* were purchased from a farm in Atibaia (São Paulo State, Brazil). The epiphyseal femoral distal cartilages were dissected out and the proteoglycans were extracted with 4M guanidine-HCl in the presence of protease inhibitors and then dialyzed against 0.4M guanidine-HCl to attain associative conditions before associative cesium chloride gradient centrifugation (21). The high buoyant density fraction (1.70mg/ml, A1 fraction) was centrifuged again under dissociative conditions to obtain A1D1 fractions. A1D1 fractions were sequentially dialyzed against 1M NaCl and water prior to subsequent analyses.

Proteoglycan monomer hydrodynamic size

Proteoglycans (300μg) were dialyzed against 0.5M sodium acetate at pH 8.0 and subjected to gel filtration on a Sepharose CL2B column (0.5x110cm) with elution using the same solution at a flow rate of 0.5mL/hour. Fractions (1mL) were assayed for their content in sulfated GAGs by the dimethylmethlylene blue (DMMB) procedure (22).

Isolation of chondroitin sulfate chains and Superose 6 chromatography

Large proteoglycans were subjected to β-elimination and reduction for 24 hours at 45°C in 1M sodium borohydride in 50mM NaOH, neutralized with acetic acid on ice, and vacuum dried after addition of an equal volume of methanol (23). The residues were washed three times with 500μL of methanol to remove borate salts, dissolved in 500μL of 4M guanidine-HCl in 50mM acetate buffer (pH6.0) and subjected to chromatography on Superose 6 (1x24cm)(Pharmacia, Sweeden). The column was eluted with the same solution at a flow rate of 0.5mL/min. Fractions (0.5 mL) were collected and assayed for their content in sulfated GAGs by the DMMB assay (22).

Fluorescent derivatization of chondroitinase digestion products with 2-aminopyridine

The large proteoglycans from the A1D1 fraction (10μg of sulfated GAGs) were digested with 5mU of chondroitinase ABC (Seikagaku America, USA) in 50 μL of 100mM ammonium acetate at pH7.4 for 2h at 37°C. Released products were collected into the filtrate of prewashed MicroCon 3 filters. Ammonium acetate was removed *in vacuo*. The residue was dissolved in 500μL of water and redried. Digestion products were derivatized with 2-aminopyridine (PA) (23). PA was freshly prepared before use by adding 500mg to 100μL of glacial acetic acid, which had been heated to and maintained at 65°C, with repeated vortexing until completely dissolved. This reagent (20μL) was added to 20-100 nmol of the chondroitinase digestion products. After incubation of the mixture for 24h at 37°C, 5μL of 6M borane dimethylamine in glacial acetic acid was added and allowed to stand overnight at 37°C. Acid was removed by speed vac evaporation and samples were stored at -20°C until analysed. Mono- and disaccharide standards were also subjected to the chondroitinase digestion procedure before fluorescent derivatization. PA-derivatized unsaturate disaccharides were decomposed by treatment with 120μL of 35 mM mercuric acetate (pH5.0). Mercuric ions were removed *in vacuo* before chromatography.

HPLC separation of PA-derivatized chondroitinase digestion products

PA-derivatives (23) were dissolved in 100μL of water and passed over a 100μL bed volume of Dowex H^+ (BioRad, USA) immediately before loading on an AS4A Ion Pac column and equilibrated with 10mM sodium trifluoroacetate (pH7.0) in a Dionex AI-450 HPLC system. Samples were eluted at a flow rate of 1mL/min with a step gradient of trifluoroacetate (10mM, 6 min; 10-50mM, 4 min; 50-150mM, 17 min; 150-250 mM, 13 min; 250-500mM, 10 min.). The eluant was monitored by fluorescent detection with an excitation wavelength of 310nm and emission wavelength of 410nm, using an in-line fluorimeter (Shimadzu, Japan).

Identification of keratan sulfate (KS) and characterization of the KS–rich region

The large proteoglycans (1.0mg of sulfated GAGs) were loaded on a Sepharose CL6B column (Pharmacia, Sweden) (0.6x120cm) and eluted with 4M guanidine-HCl in 50mM sodium acetate (pH6.0) at a flow rate of 5mL/h. The peak eluted at the void volume of the column (PI), was digested with chondroitinase ABC (Seikagaku America, USA) (10mU/mg of sulfated GAG), subjected to SDS-PAGE in a 3-16% gradient gel (24) and assayed by Western blotting with the monoclonal antibodies MST1 (produced by immunization with the large proteoglycan from the hammerhead shark cartilage; non-diluted culture medium) (25) and 4-A-4 (1:1000) (26) against keratan sulfate, as described below. Another gel was run under the same conditions and stained with 0.25% alcian blue in 3% acetic acid. For the isolation of the KS-rich region, the large proteoglycan (7.5mg of S-GAG) was digested with chondroitinase ABC (10mU/mg) in 0.1M Tris-acetate buffer (pH7.3) for 18h at 37°C. The chondroitinase treated product was subsequently digested with trypsin (Sigma, USA) and then with chymotrypsin (Sigma, Saint Louis MO, USA) at concentrations of 2μg enzyme/mg of the initial sulfated GAG, for 10h at 37°C in the same buffer. The material was then chromatographed on a Sepharose CL6B column (0.6x120cm) in 0.5M sodium acetate (pH7.0) and eluted at a flow rate of 5mL/h as previously described by Heinegård and Axelsson (27). A peak containing material that showed metachromasy with DMMB and was devoid of uronic acid (as detected by the orcinol reaction) (28) was found. Fractions of this peak were pooled and electrophoresed on 3-16% gradient SDS-PAGE for 3h at 25mA, with or without prior digestion with keratanase (Seikagaku America, USA) The gel was stained with alcian blue as described above.

Deglycosylation, SDS-PAGE and Western blotting of the core protein

A1D1 fractions were deglycosylated by sequential treatment with chondroitinase ABC (proteinase free, Seikagaku America, USA) keratanase II (Seikagaku America, USA) (0.7mU/100μg of sulfated GAGs) and endo-β-glycosidase (Seikagaku America, USA) (0.7mU/100μg of sulfated GAGs). Thirty μg of the initial sulfated GAGs were subjected to SDS-PAGE on precast 4-12% gels (Novex, USA) for 90 min at 125V (24). The material was electrotransferred onto nitrocellulose using an XCELL transfer unit (Novex, USA) (29). Membranes were blocked with 5% (w/v) skim milk powder in Tris-

buffered saline (0.5M NaCl, 20mM Tris, pH 7.5), containing 0.1% Tween 20 (TST), for 1h at room temperature. Anti-ATEGQV (1:5000) (which reacts with the IgG loop of the G1 domain of the aggrecan; Kenagy, Wight and Sandy, unpublished), anti-CDAGWL (1:3000) (which detects the protein tandem repeat loops of the G1 domain, obtained from Dr. Steve Carlson), Lec-7 (raised against a peptide contained in the lectin-like motif of the G3 domain, obtained from Dr. Kurt Doege) (1:5000) polyclonal antibodies, anti-C4S-stubs (2B6 clone, 1:750) (30) and anti-C6S-stubs (3B3 clone, 1:5000) (31) monoclonal antibodies and the peroxidase-conjugated secondary antibody (1:5000) were all diluted in 1% (w/v) milk powder in TST. Immunoreactivity was revealed using the ECL detection kit (Amersham, USA) and exposure to Hyperfilm (Amersham, USA). Rat chondrosarcoma aggrecan (32) was included for comparison.

Results

The hydrodynamic size of the proteoglycan monomer and of the chondroitin sulfate chains

Figure 1 shows the chromatographs obtained for the proteoglycan monomers extracted from the bullfrog cartilage by gel filtration on Sepharose CL2B. The Kav was 0.14 and Kav range (50% of the peak) was 0 and 0.34. Chondrotin sulfate chains released by β-elimination of the proteoglycans present in the A1D1 fraction were chromatographed on Superose 6. The chains eluted as a single peak with Kav=0.40 (Figure 2).

Fluorescent HPLC analyses of chondroitinase ABC digests

Ion exchange HPLC analysis of PA-derivatized chondroitinase digestion products was done under conditions in which all internal disaccharides and non-reducing terminals were measured (33). The majority of the fluorescent products present in the digests were the sulfated Δdisaccahrides (unsaturated disaccharide) (97.4%) (Figure 3A and Table 1). The ΔDi4S:ΔDi6S ratio was 1.6. GalNAc4S, GalNAc4,6S and the saturated disaccharides GlcA-GalNAc4S (Di4S) and Glc-GalNAc6S (Di6S) were identified as the non-reducing termini of the CS chains (Figure 3) and corresponded to

2.6% of the total chondroitinase digestion products. The average number of repeating disaccharides per chain was estimated by the ratio of interior Δdisaccharides to non-reducing termini (Table 1). The analyses also showed that sulfated GalNAc residues were the most abundant termini in the bullfrog chondroitin sulfate chain, representing 96% of the total non-reducing termini (Figure 3B and Table 1) in the form of GalNAc4S and GalNAc4,6S, which corresponded to 59% and 37%, respectively. Both Di4S and Di6S termini were present in equal amounts (2% each of the total) (Figure 3B and Table 1), and hence 4% of the chondroitin sulfate chains terminated as GlcA.

Identification of KS and characterization of the KS-rich region

The whole guanidine-HCl extract was chromatographed on Sepharose CL6B (Figure 4A). The peak eluted at the void volume was treated with chondroitinase ABC. The digestion products were electrophoresed in SDS-PAGE. Components with high relative molecular mass were seen after staining with alcian blue. The non-digested proteoglycan was in the stacking gel and the chondroitinase ABC treated proteoglycan migrated as a high molecular mass component at the top of the separating gel (Figure 4B). Other samples were electroblotted onto nitrocellulose membranes and incubated with the monoclonal antibodies MST1 (Figure 4C) or 4-A-4 (Figure 4D). Immunoreactivity for both MST1 and 4-A-4 was found in high molecular mass components only after chondroitinase digestion. Non-digested proteoglycans showed reaction with neither of the monoclonal antibodies. To isolate the keratan-sulfate rich region, the monomers of A1D1 were digested first with chondroitinase ABC and then with a trypsin/chymotrypsin combination. The digestion products were chromatographed on a Sepharose CL6B column (Figure 5A). The peak detected by the methacromasy with DMMB was devoid of uronic acid, as detected by the orcinol reaction. This product migrated as a polydisperse band centered at 110kDa in a 3-16% gradient SDS-PAGE, after alcian blue staining (Figure 5B). This alcian blue positive band was digested by keratanase II (Figure 5B).

Identification of G1/G2, G3, chondroitin 4-sulfate (C4S) stubs and chondroitin 6-sulfate (C6C) stubs on large proteoglycan

The reactivity of the large proteoglycan core protein to anti-ATEQV, anti-CDAGWL, anti-Lec-7, anti-chondroitin 4-sulfate stubs (2B6) and chondroitin 6-sulfate stubs (3B3) was examined by Western blot analyses. The anti-ATEGQV antibody detected three species with high molecular mass (300, 190 and 180kDa) in A1 fractions (Figure 6, lane 3) and a single band at about 300kDa in the A1D1 fraction (Figure 6, lane 4). The anti-CDAGWL detected at least five species with molecular mass between 130 and 300kDa in the A1 fractions (Figure 7, lane 3). The A1D1 fraction presented the same molecular species in different amounts and a further 250kDa band, besides the 300kDa full core protein. A variety of molecular species reactive to the Lec-7 antibody was found in the A1 fraction. The 300kDa component showed strong immunoreactivity to this antibody (Figure 8, lanes 3 and 4). Immunoreactivity to the 3B3 and 2B6 monoclonal antibodies was shown for many bands in the A1 and A1D1 fractions obtained by ultracentrifugation. Three main species with 60, 75 and 100kDa were found in the A1 fraction (lane 3 in Figures 9 and 10). Other bands in the A1 and A1D1 fractions migrated between 130 and 250kDa (Figure 9 and 10, lanes 3 and 4). The 300kDa band also showed reactivity to these two antibodies.

Discussion

The bullfrog epiphyseal cartilage shows a unique morphology and a distinctive role in long bone growth, as compared to the mammalian and avian models (19). We used immunochemical and biochemical assays for the identification of structural and compositional characteristics of the large proteoglycan found in the bullfrog cartilage, as compared to the classical model for aggrecan.

Chondroitin sulfate chains were isolated and shown to be about 38 disaccharides long and to have ΔDi4S as the predominant form of sulfation (ΔDi4S: ΔDi6S=1.6). Considering the number of internal disacharides, the tetrasaccharide linkage and 300kDa as the mean molecular mass for the non-reducing terminal (35, 23), it appears that the CS chains in the young adult bullfrog cartilage have a molecular mass of about 19,524Da.

Since ΔDiOS is not detected by the HPLC procedure employed in this work, it is possible that these chains are longer. However, the presence of nonsulfated disaccharides needs to be confirmed by complementary methodology.

These structural characteristics of the CS chains correspond to those found in young human aggrecan, with respect to the chain length, and to the aged human aggrecan, with respect to the structure of the non-reducing terminal, considering the presence of GalNAc4,6S, which is only found in the older individuals (33). It is worth mentioning that GalNAc4S is the only form of GalNAc at the non-reducing terminal of CS on the newborn and young human aggrecan (33).

Keratan sulfate was also identified as a component of the bullfrog large proteoglycan, by the use of two monoclonal antibodies (4A4 and MST1 clones) on Western blots. Antibody reactivity is hindered by the presence of the CS chains, as indicated by the fact that the reaction with either antibody is only achieved after chondroitinase treatment. The isolation of a KS-rich region suggests further similarity with the mammalian aggrecan. We have shown, by SDS-PAGE, that the KS-rich region is about 110kDa. This is really close to the 122kDa determined for the KS-rich region of the bovine aggrecan (27). Though it is well known that KS concentrates in a KS-rich region after the G2 domain and before the CS substitution region in all aggrecan molecules already described, except for the rat chondrosarcoma aggrecan, which lacks this KS-rich region (36, 37), its exact location on the bullfrog molecule has still to be determined.

The use of different antibodies has revealed important aspects of the large proteoglycan structure. The 2B6 and 3B3 antibodies, besides showing the existence of C4S and C6S stubs in the core protein after chondroitinase digestion, also revealed that the deglycosylated full core protein is about 300 kDa. On the other hand, the positive reactions to the anti-ATEGQV and Lec-7 antibodies are strong evidences for the existence of G1 and G3 domains. Though CDAGWL is a sequence found in G2, it is also present in G1 and, as such, cannot be used as an indicative of the existence of this globular domain. However, transmission electron microscopy of rotary shadowed isolated molecules has demonstated morphologically the existence of the G2 domain besides G1 and G3 (Covizi DZ et al, manuscript in preparation).

The presence of molecular species with molecular mass below 300kDa may represent degradation products containing the globular domains (38, 39). Since protease inhibitors were added to the extracting buffer, we do not consider the possibility of artifactual degradation of the proteoglycan monomer during extraction. However, whether they represent normal catabolic products of the aggrecan remains to be ascertained.

The aspects of the large proteoglycan of the bullfrog epiphyseal cartilage presented in this paper allow for the suggestion that it is aggrecan. Furthermore, they also reveal a high conservation of its domain structure and glycosaminoglycan substitution and that the unique characteristics of the bullfrog epiphyseal cartilage are not attibutable to differences in its aggrecan structure.

A study of the variations of aggrecan structure in different regions of the bullfrog epiphyseal cartilage, with special attention to the growth cartilage, is being considered for the near future.

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Table 1 - Quantitative data on CS chain internal and non-reducing terminal disaccharide composition.

	Total	**Non-reducing terminals**				
ΔDi4S/ΔDi6S	**Terminals**	GalNAc4S	GalNAc4,6S	Di4S	Di6S	**CS chain size[a]**
1.6	0.49 (2.6%)	0.29 (59%)	0.18 (37%)	0.01 (2%)	0.01 (2%)	~38

[a] Expressed as the number of disaccharides. This value was calculated from the ratio between the amount of internal Δdisaccharides to the amount of non-reducing terminals.

Legends

Figure 1 - Hydrodynamic size of large proteoglycan monomer. Gel filtration of proteoglycan monomers present in A1D1 fractions of the ultracentrifugation. Material present in A1D1 fractions were dialysed against 0.5M sodium acetate at pH 8.0 and loaded on a Sepharose CL2B column (0.6x120cm). Fractions (0.5mL) were assayed for the amount of GAGs by the DMMB procedure.

Figure 2 - Hydrodynamic size of large proteoglycan chondroitin sulfate. 250μg of large proteoglycan were treated with sodium borohydrate and NaOH to release the composing GAG chains. The released GAGs were chromatographed on Superose 6 and fractions were assayed for sulfated GAGs with DMMB.

Figure 3 - AS4A ion chromatography derivatized products from chondroitinase digested large proteoglycan. (A, B) The samples were chondroitinase ABC digested, derivatized and portions (200ng) chromatographed on AS4A HPLC. Column effluents were monitored for fluorescence (B) Other portions of derivatized products were treated with mercuric acetate prior to HPLC fractionation and monitoring. Products were identified by comparison of their elution times relative to standards (1, GalNAc4S-PAr; 2, GalNAc 4,6S-PAr; 3, ΔDi4S-PAr; 4, ΔDi6S-PAr; 5, Di4S-PAr; Di6S-PAr).

Figure 4 - Identification of keratan sulfate on large proteoglycan. (A) Chromatography on Sepharose CL6B of large proteoglycans total extracts. PI, peak eluted at the void volume. (B) SDS-PAGE plus alcian blue staining of the large proteoglycans present in PI. The large proteoglycan found in the stacking gel was digested by chondroitinase ABC and was retained at the top of the separating gel. (C, D) Immunochemical identification of keratan sulfate in large proteoglycans using the MST1 and 4A4 monoclonal antibodies, respectively.

Figure 5 - Characterization of keratan sulfate-rich region on large proteoglycan. (A) Sepharose CL6B of the products obtained by sequential treatment with chondroitinase ABC, trypsin and chymotrypsin. A KS-rich region was identified by the presence of

sulfated GAG (DMMB reaction – A525) and the lack of reaction for uronic acid (orcinol reaction – A670). (B) SDS-PAGE of the material present in the peak corresponding to the KS-rich region prior and after keratanase digestion. Keratanase treatment eliminates the polydisperse band with molecular mass centered at 110kDa. Alcian blue staining.

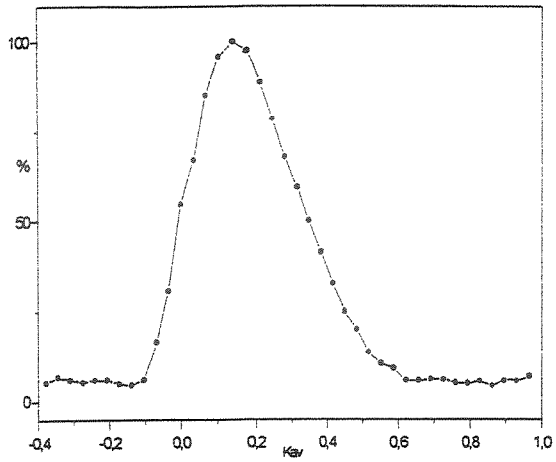
Figure 6 - Western blot of A1 and A1D1 proteoglycans with the anti-ATEGQV antibody. A1(3) and A1D1(4) extracts were deglycosylated, electrophoresed on 4-12% gradient gels and blotted to nitrocellulose for the reaction with anti-ATEGQV. 1, molecular mass markers. 2, rat chondrosarcoma aggrecan.

Figure 7 - Western blot of A1 and A1D1 proteoglycans with the anti-CDAGWL antibody. A1(3) and A1D1(4) extracts were deglycosylated, electrophoresed on 4-12% gradient gels and blotted to nitrocellulose for the reaction with anti-CDAGWL. 1, molecular mass markers. 2, rat chondrosarcoma aggrecan.

Figure 8 - Western blot of A1 and A1D1 proteoglycans with the Lec-7 antibody. A1(3) and A1D1(4) extracts were deglycosylated, electrophoresed on 4-12% gradient gels and blotted to nitrocellulose for the reaction with Lec7. 1, molecular mass markers. 2, rat chondrosarcoma aggrecan.

Figure 9 - Western blot of A1 and A1D1 proteoglycans with the 3B3 antibody. A1(3) and A1D1(4) extracts were deglycosylated, electrophoresed on 4-12% gradient gels and blotted to nitrocellulose for the reaction with the 3B3 monoclonal antibody. 1, molecular mass markers. 2, rat chondrosarcoma aggrecan.

Figure 10 - Western blot of A1 and A1D1 proteoglycans with the 2B6 antibody. A1(3) and A1D1(4) extracts were deglycosylated, electrophoresed on 4-12% gradient gels and blotted to nitrocellulose for the reaction with the 2B6 monoclonal antibody. 1, molecular mass markers. 2, rat chondrosarcoma aggrecan.



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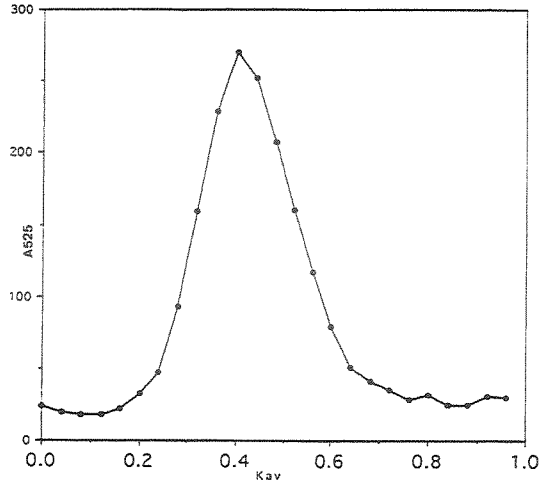

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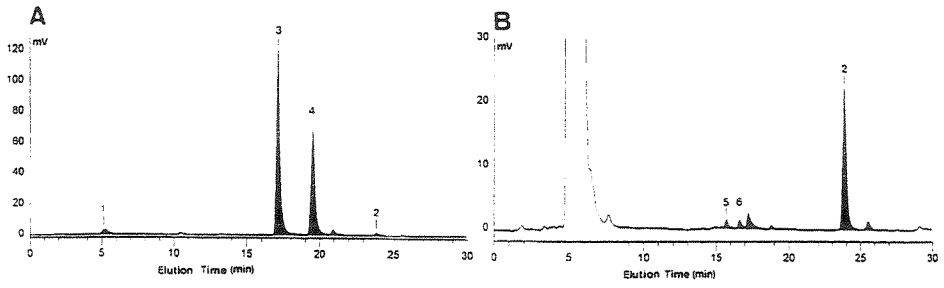

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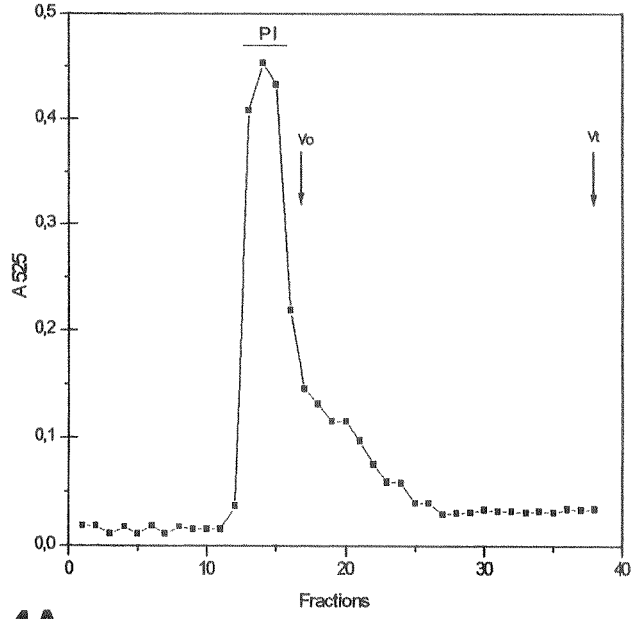
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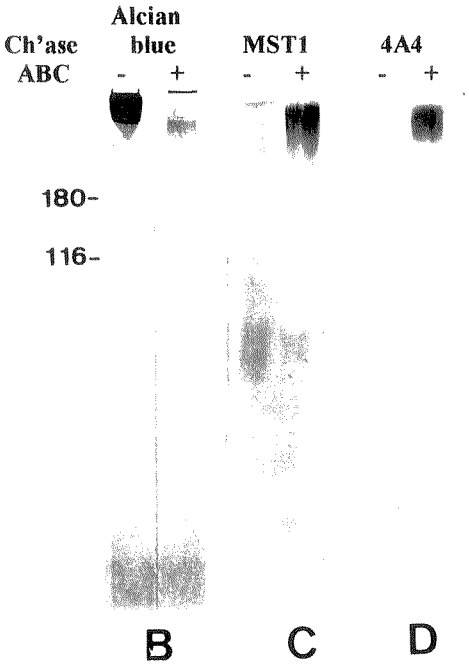
4A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180-
116-
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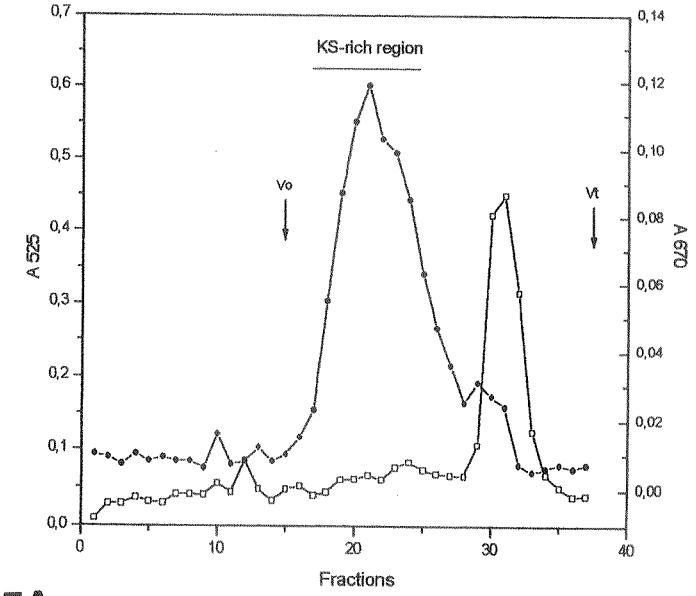
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A 525
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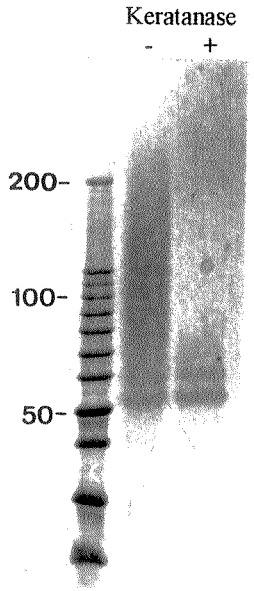
Keratanase
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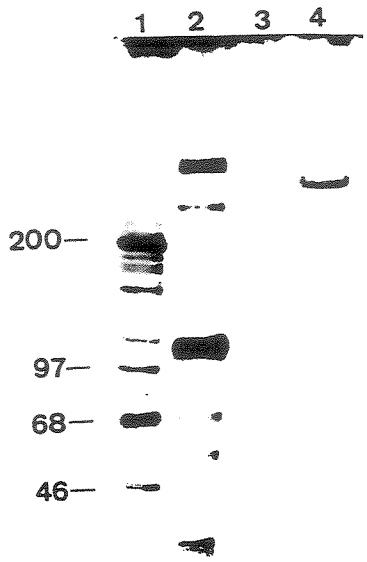
B



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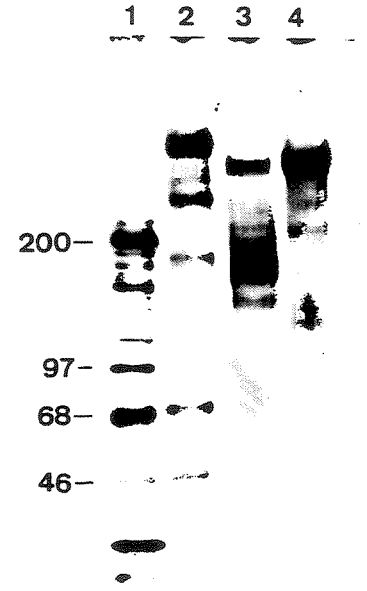

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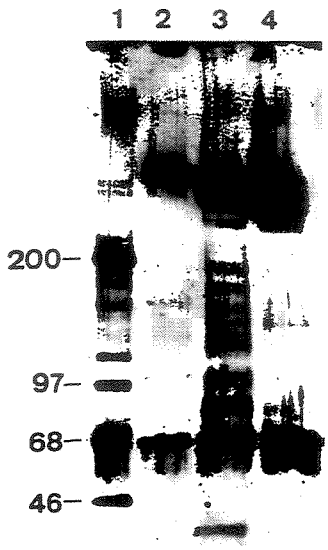

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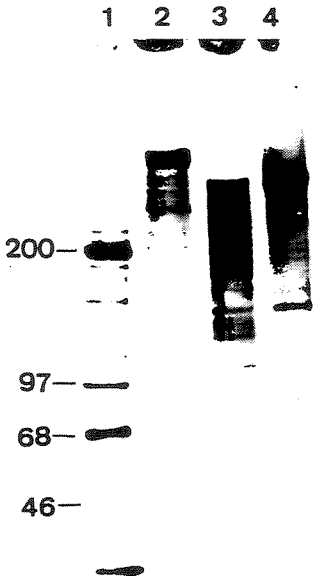

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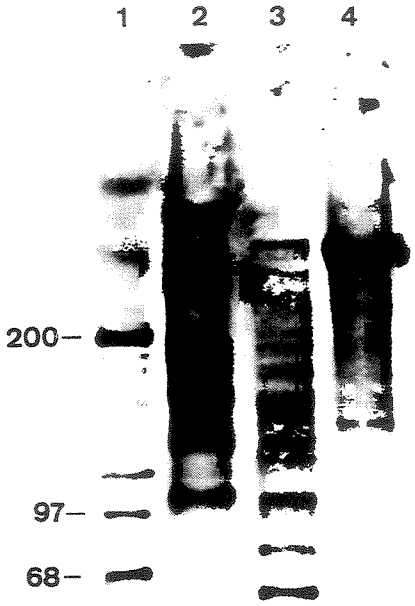
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Amphibian aggrecan: Core protein analyses and regional aspects of glycosaminoglycan substitution in the bullfrog

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Running title: Fine structure of the amphibian aggrecan

Key words: aggrecan, amphibian, chondroitin sulfate, core protein, keratan sulfate

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Abstract

A large proteoglycan was isolated from the articular, lateral and growth regions of the femoral distal cartilage of the bullfrog, *Rana catesbeiana*, and identified as aggrecan by a series of biochemical and immunochemical assays. This proteoglycan was shown to contain the three globular domains G1 and G2 (spaced 21nm from each other) and G3, and to aggregate with hyaluronan and bovine link protein. The core protein was prepared after chondroitinase and keratanase digestion and visualized by Western blotting with the aggrecan domain specific antibodies anti-ATEGQV (G1), anti-CDAGWL (G1/G2) and Lec7 (reactive with the lectin-like domain of G3). All three antibodies reacted with a predominant species that migrated with an apparent molecular size of 300kDa. Lower molecular weight immunoreactive species (~ 110 kDa) were also seen in all regions, suggesting the existence of aggrecan degradation fragments. The bullfrog aggrecan was substituted with both chondroitin and keratan sulfate chains. Aggrecan from articular and lateral cartilages contained CS chains that were composed of an average of 42 disaccharide units, and an average ΔDi0S:4S:6S sulfation isomer composition of about 11:64:24. These chains contained mostly either GalNAc4S (59% and 64%, respectively) or GalNAc4,6S (37% and 34%, respectively) at the non-reducing terminal. CS chains on the aggrecan from the growth region were distinct with respect to size and sulfation isomer composition. They had ~52 dissaccharide units and contained a higher proportion of unsulfated and 6-sulfated disaccharides. Few chains contained the disulfated GalNAc4,6S termini, as more chains terminated in GalNAc4S. Keratanase II digestion generated products that were separated by fluorophore-assisted carbohydrate electrophoresis. For all three cartilage regions, the digestion products were ~80% Gal-GlcNAc6S and ~20% Gal6S-GlcNAc6S. These data demonstrate the evolutionary conservation of the aggrecan structure in a lower vertebrate and suggest a high dependence of its functions on the domain organization.

Introduction

The cartilage extracellular matrix is a resilient composite capable of resisting tension and pressure forces. Besides its supportive function, cartilage also plays important roles in animal development as it serves as the anlage for the growth of long bones. The cartilage of the growth plate shows some distinctive compositional and histological aspects as compared to the articular cartilage. In the mammalian growth plate, the coordinated interplay between the proliferation and hypertrophy of chondrocytes and special arrangements of the extracellular matrix result in vertical expansion of the cartilage and longitudinal growth of the bone. The anuran cartilage was shown to differ from the mammalian and avian models in many respects. In these animals there is no secondary center of ossification (and thus the term growth plate does not apply), cells are not arranged in columns and there is no longitudinal collagenous septa. Furthermore, cell hypertrophy is not associated with matrix calcification and endochondral ossification is resquicial or absent during development and growth (Haines, 1942; Dickson, 1982; Felisbino and Carvalho, 1999). We have studied the bullfrog epiphyseal cartilage and concluded that the growth of long bones is mostly dependent on periosteal ossification and that the contribution of the growth cartilage (not growth plate) is only to the lateral expansion of the bone (Felisbino and Carvalho, 1999). These discrepancies between the growth of long bones in mammalian models and that of the anurans, led us to believe that a detailed study of the macromolecules building up the anuran cartilage could reveal both important aspects of the evolutionary conservation of extracellular matrix components and possible explanations for the differences in long bone growth.

Aggrecan, the large proteoglycan initially described in cartilage, consists of a core protein to which the glycosaminoglycans chondroitin sulfate and keratan sulfate, and N- and O-linked oligosaccharides are attached (Wight et al., 1991). The glycosaminoglycans have a high fixed negative charge density conferring to the aggrecan the characteristic osmotic activity (Carney and Muir, 1988). Therefore, it is assumed that aggrecan provides the tissue with the ability to withstand compressive forces and to distribute load

(Buchwalter et al., 1988). Aggrecan has a multi-domain core protein, consisting of three globular domains (G1 and G2, at the N-terminus, and G3, at the C-terminus) and at least two extended domains (the interglobular domain, between G1 and G2, and the glycososaminoglycan substitution domain, between G2 and G3) (Hascall, 1988; Iozzo 1998). The glycosaminoglycan-bearing regions have 20-30 keratan sulfate chains (Antonsson et al., 1989) and 100 chondroitin sulfate chains (Paulson et al., 1987). Chondroitin sulfate substitution takes place in two adjacent domains of the core protein (CS1 and CS2), which differ in the density of their glycosylation sites.

Further complexity in aggrecan structure is achieved by a varied sulfation pattern in both keratan sulfate and chondroitin sulfate. Such sulfation heterogeneities might influence the overall physicochemical properties of the glycosaminoglycan chains and thus provide a mechanism by which the extracellular matrix can fulfill the range of functional demands during the normal operation of a given cartilage.

Since aggrecan function is essential to the physiology and mechanical properties of cartilage we decided to characterize its structure in the bullfrog, *Rana catesbeiana*, testing the evolutionary conservation of the domain structure observed in mammalian and avian species, and a possible relationship with the above mentioned peculiarities of the anuran epiphyseal cartilage. Thus, this work presents results on the fine structure of the core protein and the attached glycosaminoglycans of amphibian aggrecan and the data confirm the evolutionary conservation of its domain structure.

Materials and methods

Materials

One-year-old bullfrogs, *Rana catesbeiana*, were reared in the town of Atibaia, São Paulo State, Brazil, for food marketing. Aggrecan from human cartilage at different ages was prepared as described before (Plaas et al., 1997) and used for comparison in some experiments. Chemicals were molecular grade and were purchased from Sigma Chemical (Saint Louis, MO) or JT Baker. Chondroitinase ACII (*Arthrobacter aurescens*), chondroitinase ABC (protease free, *Proteus vulgaris*), keratanase II, endo-β-galactosidase

(*Escherichia freundii)* were from Seikagaku America. Trypsin and chymotrypsin were from Sigma Chemical (Saint Louis, MO). Hyaluronan (Healon), Superose 6, Sephacryl S1000, Sepharose CL6B and Sepharose CL2B were from Pharmacia (Piscataway, NY). AS4A column and CarboPac PA1 column was from Dionex (Sunnyvale, CA). HPLC were carried out in a Dionex AI-450 system. The ECL reagents and the Hyperfilm were from Amersham. X-Omat film was from Kodak. Pre-cast gradient gels were from Novex Co (San Diego, CA).

Preparation of the Large Proteoglycan from the Bullfrog Epiphyseal Femoral Distal Cartilage

The epiphyseal femoral distal cartilage was excised and divided into three regions: articular, lateral and growth cartilage, as described by Felisbino and Carvalho (1999) (Fig. 1). For some experiments the different regions were pooled. The proteoglycans were solubilized by a 24h extraction with 4M guanidine-HCl buffered with 50mM sodium acetate, pH 5.8 (Heinegård, 1972). The extracts were subjected to dissociative cesium chloride gradient centrifugation (Heinegård and Sommarin, 1987) and the high buoyant density fraction (1.40mg/mL; D1 fraction) was analyzed. In another set of experiments the extracts were dialyzed against 0.4M guanidine-HCl to attain associative conditions and subjected to associative cesium chloride gradient centrifugation (Heinegård and Sommarin, 1987). The high buoyant density fraction (1.70mg/mL; A1 fraction) was centrifuged again under dissociative conditions to obtain A1D1 fractions. A1D1 fractions were sequentially dialyzed against 1M NaCl and then water, prior to subsequent analysis. Total sulfated GAGs (S-GAG) were determined by the dimethylmethylene blue assay (DMMB) (Farndale *et al.*, 1986).

Proteoglycan monomer hydrodynamic size

D1 proteoglycans from each region of the bullfrog cartilage were dialyzed against 0.5M sodium acetate at pH 8.0 and subjected to gel filtration on a Sepharose CL2B column (0.5x110cm) with elution using the same solution at a flow rate of 0.5mL/hour. Fractions (1mL) were assayed for their content of S-GAG by the DMMB assay.

Sephacryl S-1000 Chromatography

To assess the aggregability of the large proteoglycan from the bullfrog cartilage, portions from the A1D1 fraction (200μg S-GAG) were incubated with hyaluronan (Healon) (4%, w/w) and bovine link protein (4%, w/w) for 2 h at 4°C (Sandy and Plaas, 1989), before chromatography on a Sephacryl S-1000 column (24x1cm) eluted with 0.5M ammonium acetate, pH8.0 plus 0.02% sodium azide (Plaas and Sandy, 1993). Fractions (0.5mL) were collected and assayed for S-GAG by the DMMB assay.

SDS-PAGE of Core Protein and Western Blotting

Portions (30 μg) of D1-proteoglycan from each region were digested sequentially with proteinase-free chondroitinase ABC (0.7μU/μg of S-GAG) and keratanase II (0.7μU/μg S-GAG) and endo-β-galactosidase (0.7μU/μg S-GAG as described by Sandy et al. (1995). The digests were mixed with sample buffer, electrophoresed on precast 4-12% gradient gels and electroblotted onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) milk powder in Tris-buffered saline (0.5M NaCl, 20mM Tris, 0.1% (v/v)Tween 20, pH 7.5) for 1h at room temperature and then developed with primary antibodies, anti-ATEGQV (1:5000) and anti-CDAGWL (1:3000) followed by peroxidase-conjugated secondary antibody (1:5000) all diluted in 1% (w/v) milk powder in Tris-buffered saline. Immunoreactive core protein was visualized by the ECL detection kit and exposure to Hyperfilm or Kodak X-Omat film.

Rotary shadowing

An A1D1 aggrecan sample was dialysed against water and vacuum dried. To this dried material, 0.1M ammonium acetate was added to achieve a final concentration of 200μg/mL and the solution sprayed onto freshly cleaved mica and shadowed with platinum and carbon (Morris *et al.*, 1986). Carbon replicas were examined in a Phillips 410 LS transmission electron microscope at a magnification of X 54,800.

Chondrotin Sulfate Analyses

D1 fractions (250μg) were subjected to β-elimination and reduction for 24 hours at 45°C in 1M sodium borohydride and 50mM NaOH. The glycosaminoglycans were resuspended in

500µl of 4M guanidine-HCL in 50mM sodium acetate, pH7.0 and subjected to chromatography on Superose 6 (1.0x30cm) (Deutsch *et al.*, 1995; Plaas *et al.*, 1997). The fractions (500µL) were collected at a flow rate of 0.5mL/min. and assayed for their sulfated glycosaminoglycan contents by the DMMB assay. Other portions (50µg) of the D1 fractions were digested with chondroitinases, the mono- and disaccharide products fluorotagged with 2-aminopyridine and separated and quantified by anion-exchange chromatography on an AS4A column (Plaas *et al.*, 1997). To determine the proportion of ΔDiOS in the chondroitinase digests, additional portions (50µg) of the large D1 proteoglycan were digested with chondroitinase ABC and ACII. The products were reduced with sodium borohydride, chromatographed on a CarboPac PA1 ion-exchange column in a Dionex AI-450 HPLC system and unsaturated disaccharides quantified by pulsed electrochemical detection (PED) (Midura *et al.*, 1994). The proportion of CS to KS was determined by hexosamine compositional analysis of the HCl hydrolyzed proteoglycan using a CarboPac PA1 column (Plaas *et al.*, 1996).

FACE Analyses of Keratanase II Digestion Products

Keratanase II digestion of the large D1 proteoglycan was done in 100mM ammonium acetate, pH6.0. Usually, 20µg of sulfated glycosaminoglycans were digested in 50 µl of buffer with 2.5mU of keratanase II for 26h at 37°C. The samples were dried by speed vac and incubated with 5µl (0.5mg/20µL of 3:17 glacial acetic acid/dimethyl sulfoxide) of 2-aminoacridone and 5µl (2.5mg/40µL of water) of sodium cyanoborohydride at 37°C for 16h (Jackson, 1994). The labeled samples were cooled on ice and 30µl 25% glycerol added at 4°C. The products were analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) (Monogel - Glyko) for 1h at 500mV, analyzed using a Stratagene Eagle Eye equipment and quantified by determining the pixel densities in the separated fluorescent bands using NIH Image Analysis software.

Results

Monomer Hydrodynamic Sizes

Figure 2 shows the chromatographs obtained for the proteoglycan monomers extracted from the different regions of the bullfrog cartilage after gel filtration in Sepharose CL2B. The Kav (peak) and Kav range (50% of the peak) for the articular, lateral and growth regions were 0.14 (-0.02/0.36), 0.17 (0.02/0.36) and 0.10 (0/0.28), respectively. At the peak, the monomers of the growth region were slightly larger than those from the other regions. Furthermore, the monomers of the articular region were more polydisperse than those of the other regions, and the smaller monomers of the growth region were larger than the smaller monomers of the other regions.

Aggregation Properties

The ability to form aggregates with hyaluronan was assessed by mixing the large proteoglycan from A1D1 fraction of the bullfrog cartilage with hyaluronan and with hyaluronan plus bovine link protein. The large proteoglycans of the bullfrog cartilage were able to aggregate with hyaluronan both with and without added bovine link protein (Fig. 3). The presence of the link protein increased the size of the aggregates by recruiting a larger number of monomers, showing the ability of the bullfrog large proteoglycan to form complexes with the bovine link protein.

Identification of the Large Proteoglycan Core Protein

The high buoyant density chondroitin sulfate-proteoglycan of the three regions of bullfrog epiphyseal femoral distal cartilage was identified as a "mammalian-type" aggrecan on the basis of multiple immunoreactivities of the core protein on Western blots (Fig. 4-5). These fractions contained a single core species of about 300kDa which reacted with anti-ATEGQV and anti-CDAGWL. The apparent size of the core protein was markedly smaller than those of the rat (about 350kDa), bovine and human (about 400kDa).

Ultrastructure of Rotary Shadowed Molecules

The large proteoglycans present in the A1D1 fraction obtained by ultracentrifugation, were examined using the transmission electron microscope after rotary shadowing. The images

of the large proteoglycans (Fig. 6) showed the existence of three globular domains. G2 was 21nm apart from G1 and the glycosaminoglycan substitution concentrated on the core protein region immediately before G3.

GalNAc / GlcNAc ratios

The ratio between the amounts of n-acetylgalactosamine and n-acetylglucosamine was 12 for the articular region, 13 for the lateral region and 10 for the growth region. Glycosylation of the large proteoglycan in terms of the proportion between CS and KS in the three regions of bullfrog cartilage was thus very similar (Fig. 7).

Hydrodynamic Size of the Chondroitin Sulfate Chain

Chondroitin sulfate chains released from the large proteoglycans purified from the three regions of bullfrog cartilage were chromatographed on Superose 6 (Fig. 8, Table II). Chains from articular, lateral and growth regions eluted with a Kav=0.42. However, a larger chondroitin sulfate chain population was detected in the growth cartilage (Kav=0.35), indicating a heterogenous hydrodynamic size distribution in this region.

Sulfation of Reducing Internal Residues on Chondroitin Sulfate

The unsaturated disaccharides derived from chondroitinase digestion of chondrotin sulfate were chromatographed on CarboPac PA1 and unsaturated disaccharides quantified by pulsed electrochemical detection. The unsaturated composition of the large proteoglycan chondroitin sulfates isolated from articular, lateral and growth regions were similar to each other (Table I). The most abundant ΔDisaccharide in all three regions was ΔDi4S. The amount of ΔDi4S was greater in the articular and lateral cartilages. The ratios of ΔDi6S:ΔDi4S in the articular, lateral and growth cartilage were 0.4, 0.4 and 0.7, respectively. The amount of ΔDiOS in the growth cartilage was higher than in the other two regions.

Sulfation of Non-Reducing Terminal Residues and the Average Number of Internal Disaccharides in the Chondroitin Sulfate chains

Ion exchange HPLC analyses of fluorotagged chondroitinase digestion products was done under conditions in which the non-reducing terminal monosaccharides and disaccharides could be quantified. The majority of the fluorescent products present in the digests of the large proteoglycan from articular, lateral and growth cartilage were either GalNAc4S or GalNAc4,6S (Table I). The ratios of GalNAc4,6S:GalNAc4S in the articular, lateral and growth cartilage were 0.7, 0.5, and 0.5, respectively. The non-reducing termini of the chondroitin sulfate chains GlcA-GalNAc4S (or Di4S) and GlcA-GalNAc6S (or Di6S) (saturated disaccharide) were identified in the digests and constituted 4%, 4% and 5% of the total chondroitinase digestion products in the articular, lateral and growth regions of the bullfrog cartilage, respectively. In the articular cartilage, the proportion of chains terminating with GalNAc4,6S was significantly increased as compared to the growth or lateral cartilages. The average number of repeating disaccharides per chain was calculated from the ratio of interior (unsaturated disaccharides) to non-reducing termini (Table II). The chondroitin sulfate chain size was identical in articular and lateral cartilages (~42 disaccharides). In the growth cartilage, the CS chain size was longer, with about 56 disaccharides.

Sulfation of the Keratan Sulfate Chains

The products digested with keratanase II and labeled with 2-aminoacridone and sodium cyanoborohydride were analysed by fluorophore-assisted carbohydrate electrophoresis (FACE) and quantified by determining the pixel densities in the separated fluorescent bands (Fig. 9; Table III). Keratan sulfate disaccharide in the three cartilages showed an identical sulfation pattern. The amount of monosulfated disaccharides was about four-fold higher than that of the disulfated disaccharides.

Discussion

The structural study and the immunochemical assays done in this work allow for the conclusion that the large proteoglycan of the bullfrog cartilage is aggrecan, and, to our knowledge, this paper gathers the most detailed analyses for aggrecan of any given species.

The results demonstrated that antibodies raised against peptides found in the G1/G2 and G3 domains of human aggrecan cross-reacted with the bullfrog large proteoglycan, showing an immunochemical conservation between the two species. ATEGQV is a peptide found in the aggrecan G1 domain and in the link protein, but not in other large aggregating proteoglycans such as versican, brevican or neurocan (Zimmerman and Ruoslahti, 1989; Rauch *et al.*, 1992). Though CDAGWL peptide is found in both G1 and G2 domain and could not by itself demonstrate the presence of a G2 domain in the bullfrog large proteoglycan, the ultrastructural observations of the rotary shadowed molecules demonstrated the physical existence of the three globular domains and that the fact that G1 is 21nm apart from G2. Rotary shadowing also showed that glycosaminoglycan substitution concentrates towards the C-terminus before G3.

The presence of anti-ATEGQV antibody reactive bands ~110 kDa suggests that aggrecan catabolic forms may arise by proteolytic cleavage at the CS substitution region. Whether this kind of proteolytic processing is involved in some sites of cartilage calcification and/or resorption in the epiphyseal apparatus of *Rana catesbeina* is now under investigation.

The aggregating ability of the large proteoglycan was demonstrated by incubation with hyaluronan and the experiments also showed that the bullfrog aggrecan is able to form interspecies complexes with the bovine link protein, which stabilizes the aggregates.

Chondroitin sulfate is a major component of the bullfrog aggrecan. The internal disaccharide composition of the chondroitin sulfate chains was analysed, and it was shown that there was a predominance of ΔDi4S over ΔDi6S for all three regions, but the growth cartilage accumulates a greater amount of ΔDi6S, a similar compositional aspect of the growth cartilage in bovines (Deutsch *et al.*, 1995). The growth cartilage also showed an increased amount of ΔDiOS, a characteristic of the growth cartilage in different mammalian species (Deutsch *et al.*, 1995; Plaas *et al.*, 1997).

GalNAc4S and GalNAc4,6S residues were the most abundant non-reducing termini. They were found in equal amounts in the lateral and growth cartilages, but we found an increased amount GalNAc4,6S in the articular cartilage. This increased amount of GalNAc4,6S represents a prominent similarity between the bullfrog articular cartilage and adult human cartilage, which also shows an increased amount of this terminal residue (Plaas *et al.*, 1997).

The CS chain size was similar in the articular and lateral regions with an average number of 41 and 42 internal disaccharides, respectively, as demonstrated by the HPLC analyses of the aminopyridine-derivatized chondroitinase digestion products. This number was also similar to the size reported for CS in the postnatal growth cartilage of other species (Plaas *et al.*, 1997). However, the growth cartilage showed longer CS chains, with an average of 56 internal disaccharides, correlating with the existence of a second population with Kav=0.35 in addition to the main population with Kav=0.42. This latter finding is consistent with previous reports (Weitzhandler *et al.*, 1988; Deutsch *et al.*, 1995), showing increased hydrodynamic size of the CS on the aggrecan in the hypertrophic zone of mammalian and avian growth plate cartilages. Since the growth region of the bullfrog cartilage was not divided into the composing zones, we may suppose that the longer chains arose from the deeper regions (hypertrophic zone), close to the bone marrow.

The amount of keratan sulfate was about one tenth that of CS. The KS chains in the bullfrog aggrecan showed a predominance of monosulfated disaccharides over the disulfated disaccharides. These characteristics (i.e. proportion to CS and sulfation pattern are very similar to fetal and very young human individuals (Plaas, unpublished).

From the detailed analysis of the aggrecan extracted from the different regions of the bullfrog epiphyseal cartilage carried out in this work, it turned out that the only distinctive aspects were the length and sulfation pattern of the chondroitin sulfate chains.

It has been reported that the cartilage of immature individuals presents different amounts of ΔDi4S, while being almost absent in adult specimens. Though there are evidences for an increased proportion of ΔDi4S towards the hypertrophic and calcifying zones of different species, this being correlated with the calcification and ossification processes they undergo (Mourão *et al.*, 1976; Mourão and Dietrich, 1979; Harab and Mourão, 1989), this was not confirmed for bovines (Deutsch *et al.*, 1995) or for the bullfrog

(this paper). The presence of ΔDi4S and a lower ΔDi6S/ΔDi4S ratio for the epiphyseal cartilage of the bullfrog may be indicative that these animals are relatively young. Whether this compositional aspect is also correlated to the existence of ectopic sites of calcification in these two regions (Felisbino and Carvalho, 1999) remains unclear.

While the methodological approaches employed in this work were sensitive enough to detect these modifications in aggrecan structure, the assumption that these structural variations could be responsible for the differentiation of the articular, lateral and growth cartilage is still premature. It is worth stressing that complex interactions between extracellular matrix components and cells do exist. The knowledge on the extracellular matrix of the bullfrog cartilage thus seems very important as a basis for the definition of such interactions.

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Abbreviations used: CS, chondroitin sulfate; DMMB, dimethylmethylene blue; Δdisaccharide, unsatured disaccharide; ΔDiOS, 2-acetoamido-2-deoxy-3-*O*-β-D-gluco-4-enepyronosyluronic acid; ΔDi4S, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyronosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi6S, 2-acetoamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyronosyluronic acid)-6-*O*-sulfo-D-galactose; FACE, fluorophore-assisted carbohydrate electrophoresis; GAG, glycosaminoglycan; GalNAc4S, *N*-acetylgalactosamine-4-sulfate; GalNAc4,6S, *N*-acetylgalactosamine-4,6 disulfate; KS, keratan sulfate; PA, 2-aminopyridine; PED, pulsed electrochemical detection; PG, proteoglycan; S-GAG, total sulfated glycosaminoglycans; TBS-T, Tris-buffered saline containing Tween 20.

Legends

Fig. 1. Schematic drawing of the distal femoral head of the bullfrog. The articular cartilage (1), lateral cartilage (2) and growth cartilage (3) were sampled for the analysis done in this work. PB = Periosteal bone.

Fig. 2. Gel filtration of proteoglycan monomers present in D1 fractions obtained by ultracentrifugation. Material present in D1 fractions was dialysed against 0.5M sodium acetate at pH8.0 and loaded onto a Sepharose CL2B column (0.6x120cm). Fractions (1mL) were assayed for the amount of S-GAG by the DMMB procedure.

Fig. 3. Aggregation properties of the large proteoglycan from the bullfrog epiphyseal cartilage. Proteoglycans present in A1D1 fractions (200μg of glycosaminoglycans) were analysed in Sephacryl S-1000 as monomers (▣), in the presence of 4% Healon (♦) or in the presence of 4% Healon plus 4% bovine link protein (◘). Fractions were assayed for their content in glycosaminoglycans.

Fig. 4. Western blot of proteoglycans present in D1 fractions obtained by ultracentrifugation of the Gu-HCl extracts from each of the three regions of the bullfrog cartilage with the ATEGQV antibody. D1 proteoglycans were enzymatically deglycosylated, electrophoresed on a 4-12% gradient gel and blotted onto nitrocellulose for detection with the anti-ATEGQV antibody. RCS = rat chondrosarcoma aggrecan. AC = articular cartilage. LC = lateral cartilage. GC = growth cartilage.

Fig. 5. Western blot of proteoglycans present in D1 fractions obtained by ultracentrifugation of the Gu-HCl extracts from each of the three regions of the bullfrog cartilage with the CDAWGL antibody. D1 proteoglycans were enzymatically deglycosylated, electrophoresed on a 4-12% gradient gel and blotted onto nitrocellulose for

detection with the HAL antibody. RCS = rat chondrosarcoma aggrecan. AL = articular cartilage. LC = lateral cartilage. GC = growth cartilage.

Fig. 6. Ultrastructure of the large proteoglycan of the bullfrog cartilage. The large proteoglycans from the A1D1 fractions obtained by ultracentrifugation were dialysed against water and vacuum dried. They were resuspended in 0.1M ammunium bicarbonate and sprayed onto freshly cleaved mica and rotary shadowed with platinum and carbon. The existence of the three globular domains (G1-G3) can be readly observed. The concentration of long glycosaminoglycan chains may also be observed. The distance between G1 and G2 is 21nm. The bar corresponds to 100nm. → : G1, G2 and G3 domains.

Fig. 7. Hexosamine composition of the aggrecan from amphibian and human cartilage obtained by HPLC on a CarboPac PA1 column of the HCl hydrolysed proteoglycan.

Fig. 8. Hydrodynamic size of the chondroitin sulfate chains attached to the large cartilage proteoglycan. 250µg of large proteoglycans purified from epiphyseal femoral distal cartilages were treated with alkaline borohydride to release the CS chains, which were chromatographed on Superose 6. Fractions (1mL) were assayed for metachromasy with dimethylmethylene blue.

Fig. 9. FACE analysis of the keratanase II digestion products obtained for the three regions of the bullfrog epiphyseal cartilage. The migration positions of the monosulfated and disulfated disaccharides resulting from the digestion of corneal keratan sulfated are shown. Human aggrecan cartilages: **1**, **8** – 68-year-old; **3** – fetal; **4** – juvenile (newborn to 1 year). **2** – corneal keratan sulfatte. **5** – Articular cartilage. **6** – lateral cartilage. **7** – growth cartilage.

Table I. Chondroitin sulfate composition for the different regions of the bullfrog cartilage. The internal disaccharide composition was determined by CarboPac PA1 ion exchange HPLC with PED detection. Terminal residues were determined by AS4A ion exchange chromatography of fluorescently tagged chondroitinase ABC and ACII digestion products using fluorescence detection.

Cartilage regions	**Internal disaccharides**				**Terminal residues**
	ΔDi0S	ΔDi4S	ΔDi6S	6S:4S	GalNAc4,6S: GalNAc4S
	(% of total Δdisaccharides)				
Articular	12	64	24	0.4	0.7
Lateral	10	64	25	0.4	0.5
Growth	18	49	33	0.7	0.5

Table II. Chondroitin sulfate chain size as estimated by size exclusion chromatography on Superose 6 and by the ratio between the total number of internal Δdisaccharides and the number of non-reducing termini as determined by HPLC measurements.

Cartilage type		**Kav Superose 6[a]**	**Average number of repeating disaccharides[b]**
Bullfrog	Articular	0.42	~42
	Lateral	0.42	~41
	Growth	0.42 and 0.35	~56
Human[c]	Fetal	0.40	~66
	1-year-old	0.46	~42
	68-year-old	0.62	~14

[a]Column eluted with 4M guanidine-HCl in 50mM sodium acetate, pH7.0

[b]Calculated from measurements of fluorescently tagged chondroitinase ABC and ACII digestion products using fluorescence detection.

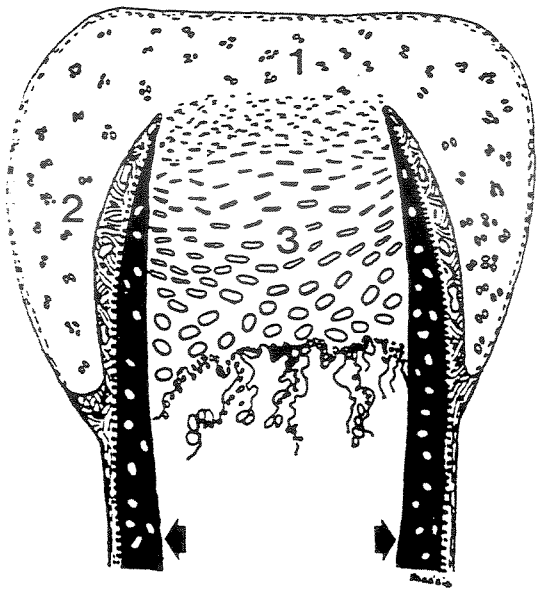
[c]From Plaas et al. (1997).

Table III. Sulfation pattern of keratan sulfate disaccharides produced by keratanase II digestion of the large bullfrog proteoglycan, as determined by FACE analyses.

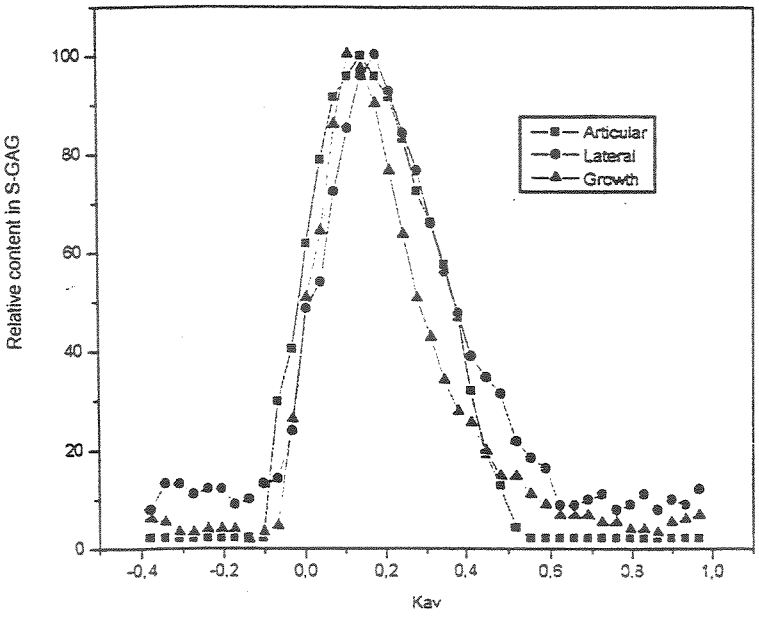
Cartilage type		Gal6S-GlcNAc6S[a]	Gal-GlcNAc6S[a]	Gal6S-GlcNAc6S: Gal-GlcNAc6S
Bullfrog	Articular	82	18	0.21
	Lateral	69	17	0.25
	Growth	81	19	0.24
Human[b]	Fetal	82	18	0.22
	1-year-old	56	44	0.78
	68-year-old	35	65	1.85

[a]As a percentage of the total disaccharides.

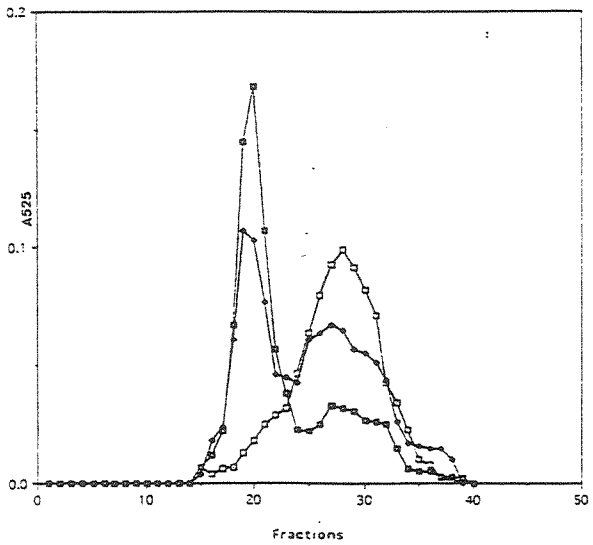
[b]From Plaas et al. (1997).



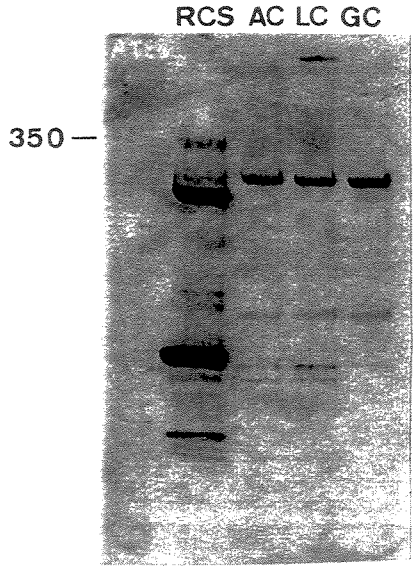
1


Relative content in S-GAG
100
80
60
40
20
0
-0,4
-0,2
0,0
0,2
0,4
0,6
0,8
1,0
Kav
Articular
Lateral
Growth

2


A525
0.2
0.1
0.0
0
10
20
30
40
50
Fractions

3



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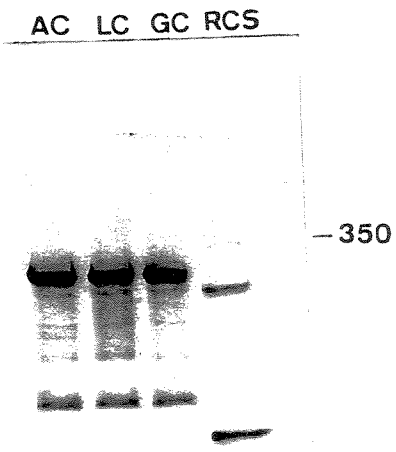

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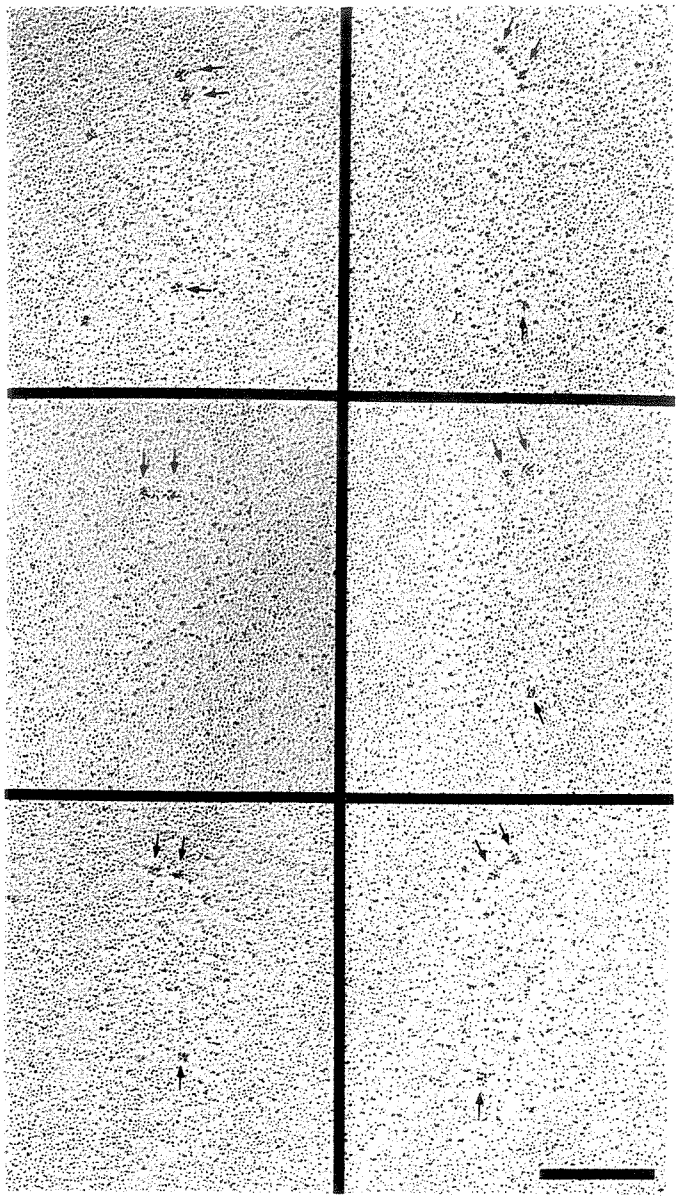
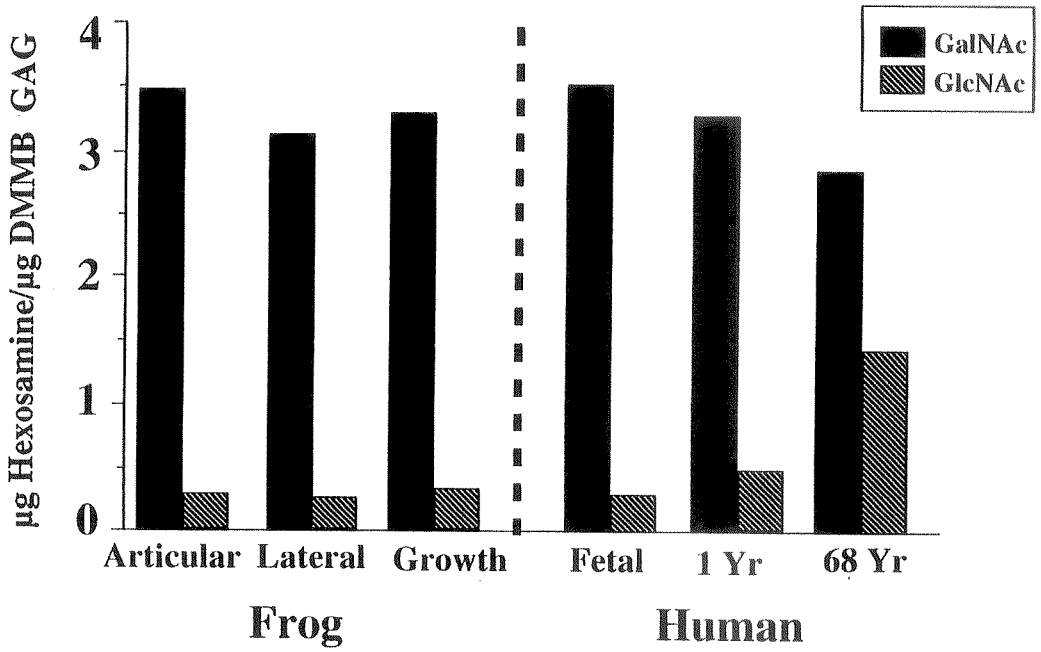
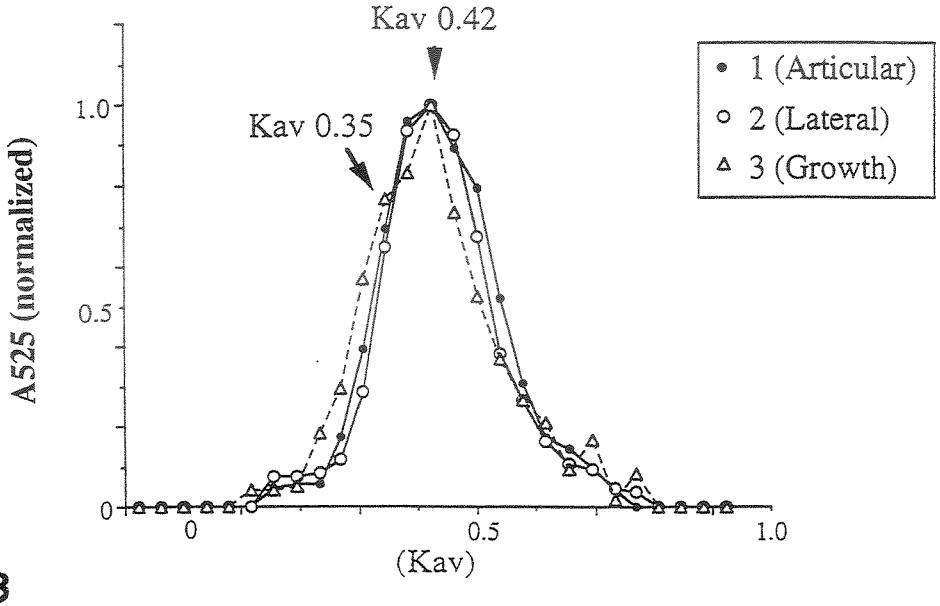


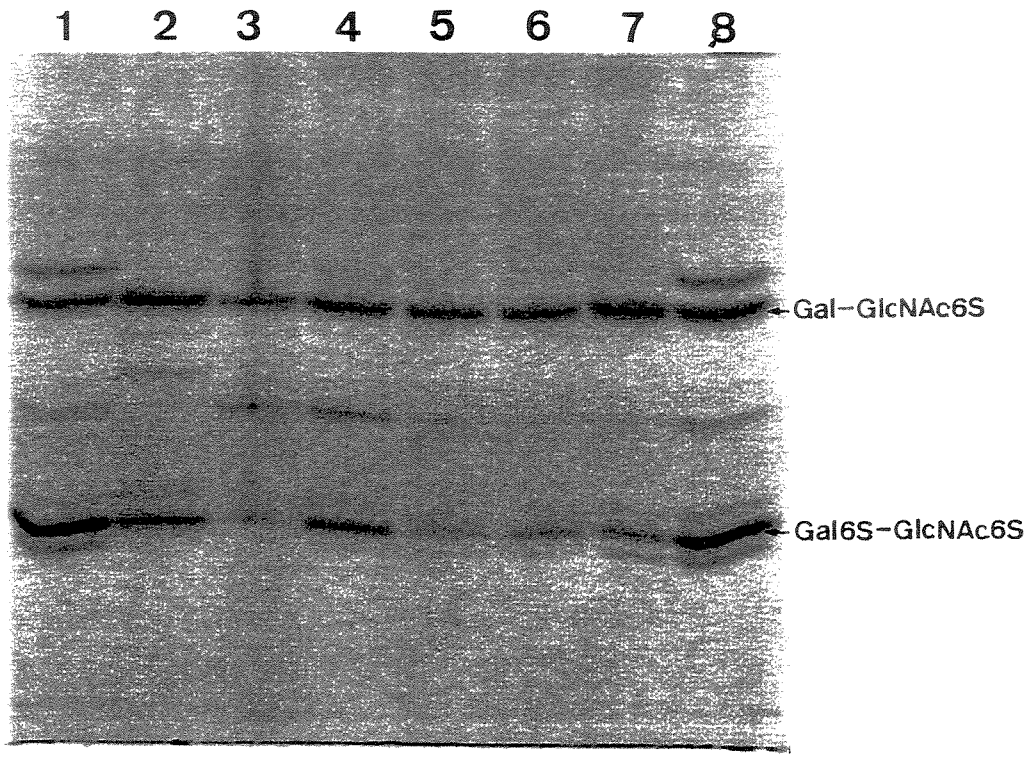
6


µg Hexosamine/µg DMMB GAG
4
3
2
1
0
GalNAc
GlcNAc
Articular
Lateral
Growth
Fetal
1 Yr
68 Yr
Frog
Human

7


Kav 0.42
Kav 0.35
1 (Articular)
2 (Lateral)
3 (Growth)
A525 (normalized)
1.0
0.5
0
0
0.5
1.0
(Kav)

8



9

Conclusões Gerais

1 – Diferenças na quantidade de colágeno (determinada pelo conteúdo em hidroxiprolina) e de proteoglicanos foram encontradas entre as três regiões da cartilagem epifisária de rãs. A cartilagem de crescimento mostrou maior hidratação com menor quantidade de colágeno e maior quantidade de proteoglicanos.

2 – Sítios de calcificação foram encontrados nas diferentes regiões da cartilagem. Diferenças quanto ao padrão de crescimento dos cristais de cálcio e na organização da matriz orgânica distinguem os sítios de calcificação da cartilagem hipertrófica daqueles considerados ectópicos (em especial na cartilagem lateral).

3 - O grande proteoglicano da cartilagem epifisária de rãs foi identificado como agrecam, semelhante ao de mamíferos e aves, com base em parâmetros imunoquímicos e bioquímicos. A massa molecular relativa do *core* protéico de rãs é menor do que do *core* protéico do agrecam de rato (aproximadamente 350kDa) e de boi e de humanos (aproximadamente 400kDa).

4 - A proporção entre condroitim sulfato (GalNAc) e queratam sulfato (GlcNAc) nas três regiões da cartilagem foi semelhante. A composição em dissacarídeos insaturados (ΔDiS) do condroitim sulfato de agrecam isolados da cartilagem articular e lateral de rãs foram similares entre si. O condroitim sulfato da cartilagem de crescimento, por outro lado, continha uma proporção mais elevada de dissacarídeos insaturados não sulfatados (ΔDiOS) e um aumento na razão de dissacarídeos insaturados 6-sulfatados e 4-sulfatados (ΔDi6S:ΔDi4S). Os dissacarídeos saturados (terminal não-redutor) presentes em maior quantidade nas cadeias de condroitim sulfato das três cartilagens foram a N-acetilgalactosamina 4-sulfatada e a N-acetilglicosamina 4,6-sulfatada. Na cartilagem articular, a proporção de cadeias terminando com N-acetilgalactosamina 4,6-sulfatada foi significantemente aumentada, quando comparada com as cartilagens lateral e de crescimento. O tamanho da cadeia de condroitim sulfato foi similar nas três regiões, e

comparáveis ao tamanho descrito para o condroitim sulfato de cartilagens de crescimento pós-natal de outras espécies. Foi detectada uma subpopulação com cadeias mais longas na cartilagem de crescimento. O queratam sulfato do agrecam de cartilagem de rãs mostrou idêntico padrão de sulfatação nas três cartilagens. A proporção de dissacarídeos monossulfatados (gal-glcNAc6S) foi cerca de quatro vezes maior de que a de dissacarídeos dissulfatados (gal6S-glcNAc6S).

5 - Análises da ultraestrutura das moléculas de agrecam por *rotary shadowing* mostrou a presença de três domínios globulares e uma região linear com as moléculas de glicosaminoglicanos ligadas. O domínio G1 estava a 21nm de distância do domínio G2, enquanto a região dos glicosaminoglicanos foi observada entre os domínios G2 e G3.

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