

Study of serum aggrecan fragments in handigodu disease precipitated by DMB dye

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Abstract

Handigodu disease is a disorder of osteoarticular system, specific to particular region viz shimoga and chickmagalore districts of Karnataka state, India. The present study focuses to understand the metabolism of aggrecan, a key glycoprotein of cartilage matrix in Handigodu Disease (HD) using serum specimen. The Dimethylmethylene Blue Chloride (DMB) dye was used to precipitate glycosaminoglycans (GAGs) associated proteins in serum. DMB dye was removed from GAGs associated proteins and electrophoresed on SDS-PAGE. The five GAGs associated protein bands were separated, among them two bands; 21.5 KDa and 55.4 KDa were immunoreactive to anti-human aggrecan antibody. These two GAGs associated protein bands were more dense (86% for 21.5 KDa and 61 % for 55.4 KDa) in affected group when compared to unaffected group in Handigodu Disease. These results may imply defective metabolism of aggrecan in a disease condition. The study needs to be extend for other arthritis / dysplastic bone disorders.

Key words: Glycosamino glycans, Aggrecan, DMB dye, Arthritis, Bone, Handigodu Disease

Introduction

The proteoglycan super family now contains more than 30 molecules that fulfill variety of biological functions. Proteoglycans acts as tissue organizers, influence cell growth and the maturation of specialized tissues. Proteoglycans also play role as biological filters and modulate growth-factor activities, regulate collagen fibrillogenesis and skin tensile strength, affect tumor cell growth and invasion, influence corneal transparency and neurite outgrowth. Additional roles derived from studies of mutant animals indicate that certain proteoglycans are essential to life whereas others might be redundant (Iozzo 1998). Proteoglycans are macromolecules,

constructed of a protein core to which many glycosaminoglycan chains are attached. Hyaluronic acid is non covalently bound to this proteoglycans aggregate. About 10% weight of proteoglycans is protein and 90% is glycosaminoglycans. Glycosamino glycans extensively constitute negatively charged chondroitin sulfate (CS) and keratan sulfate (KS) repels each other. Glycosaminoglycan electrostatic repulsion is along the chain and between the chains, therefore chains assume a fully extended conformation. Subtypes of proteoglycans include aggrecan and small proteoglycans. Aggrecan a key proteoglycan molecule in the cartilage matrix and creates the osmotic properties necessary for cartilage to resist compressive loads. Link protein is a small glycoprotein serves to stabilize non-covalent association of the aggrecan subunits with hyaluronic acid in aggregate. In the protein core approximately 100 chondroitin sulfate and 50 keratan sulfate chains are attached (Buckwalter and Rosenberg 1982; Kimura et al. 1980). The main proteoglycan component of cartilaginous tissues, aggrecan, plays important structural and biomechanical roles [Heinegard and Oldberg 1989; Buschman and Grodzinsk 1995) and partially controls biochemical processes such as matrix calcification (Poole et al. 1989). The most of the methods have been applied to study proteoglycans in tissue culture or by using radioisotopic techniques. The manuscript emphasizes on Aggrecan/proteoglycans degradation or GAGs associated proteins entered the circulatory system after their metabolism in Handigodu disease (HD). HD is a disorder of the osteo-articular system prevalent in few villages of two districts of the state of Karnataka in southern India and HD could be regarded as late onset spondylo epi (meta) physeal dysplasia. The HD has been classified mainly into three groups on the basis of X-ray analysis namely Type-I (predominantly Arthritic), Type-II (predominantly Dysplastic) and Type-III (Dwarf) (Agarwal et al. 1994).

Mallikarjun Badadani, S V SureshBabu, K T Shetty,

Materials and methods

Specimen collection

The HD affected patients clinically and x-ray radiologically confirmed have been selected for this study in the age group of 20-60 years old with their consent (Type-I; n=4, Type-II; n= 4, Type-III; n=4). The eight healthy individuals with age group of 20-60 years old from the same family members of HD affected were

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selected in this study as controls (Unaffected: n=8). All the controls clinical and by x-ray diagnosis were normal. The study has been approved by NIMHANS human ethics committee. Overnight fasting serum specimens have been collected and stored at -80°C.

Chemicals and reagents

The Dimethyl methylene blue chloride (DMB) dye has been purchased from Polysciences Inc USA. The anti-human aggrecan primary antibody was purchased from US Biologicals, USA (Cat. No.1059-53D). The antibody was raised in mouse against human cartilage aggrecan as immunogen. Other reagents used were of analytical grade.

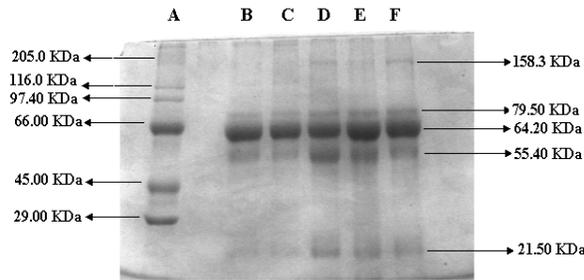


Figure 1: Typical SDS PAGE profile of GAGs associated proteins from serum. Lane A: Molecular Weight markers; Lane B and C Unaffected group (Control); Lanes D, E and F represent DMB precipitate of GAGs from serum of Type I, Type II and Type III of HD respectively. The proteins were revealed by staining with Coomassie brilliant blue dye. The serum was normalized to comparable amount of protein ($\approx 10 \mu\text{g}$ protein) loaded on to different lanes; Note the presence of intense bands for proteins corresponding to MWt 55.40 KDa and 21.50 KDa in HD affected subjects (lanes D,E and F) as compared to unaffected subjects (lanes B and C).

Electrophoresis and western blotting

GAGs precipitating dimethyl methylene blue chloride (DMB; 0.2 mM) reagent has been prepared by dissolving 6.96 mg DMB dye in 2 ml of 95% ethanol and make up to 100 ml with 0.1M Sodium formate buffer, pH 3.5. The reagent is stable for six months and stored in a brown colored bottle at room temperature. For 0.050 ml of serum in eppendorf tube, 0.500 ml of GAGs precipitating DMB reagent has been added and mixed gently. The tubes incubated at room temperature for 20 minutes to form precipitate, centrifuged at 5000 rpm for 15 min and supernatant has been siphoned off. To the precipitate, 0.100 ml of SDS(0.25%) in saline (0.085% NaCl) solution has been added and vortexed to dissolve. The 1.400 ml of ethanol (95% w/v) has been added, mixed gently and kept in an ice bucket for 30 minutes to reprecipitate. Tubes were centrifuged at 10000 rpm for 15 minutes and the supernatant was decanted. This Precipitate was dissolved in saline (0.085% NaCl) and protein concentration has been determined by Bradfords method (Bradford 1976). The 10 μg equivalent of proteins were loaded and electrophoresed by 150 Volts in SDS-PAGE (10%). The proteins were electroblotted for 2 hours on PVDF membrane at 150 mA and 15 Volts. The Immunoreaction has been carried out with mouse anti-human aggrecan primary antibody (US Biologicals); the secondary antisera used were anti-mouse alkaline phosphatase conjugated, and the substrates used were BCIP (5-Bromo-4-chloro-3-indolyl phosphate) and NBT (p-nitroblue tetrazolium chloride) for staining.

Results

The five GAGs associated proteins from serum were precipitated by

DMB dye and separated in SDS-PAGE. The lanes B and C represents unaffected group while D, E and F lanes are from HD affected (Figure 1). The two proteins 21.5 KDa and 55.4 KDa were immunoreactive to anti-human aggrecan antibody (Figure 2). The density has been calculated from Bio-Rad Gel documentation system. The average of the unaffected and the average of affected from Type I, Type II and Type III has been taken together for percent calculation. In unaffected group these two proteins were less dense i.e 86% for 21.5 KDa, 61% for 55.4 KDa in SDS-PAGE electrophoresis pattern and 80% for 21.5 KDa, 66% for 55.4 KDa for Western immunoblot pattern) than affected group (Table 1).

Data is based on the findings from three repeat experiments carried out on different specimens at different time intervals within three months.

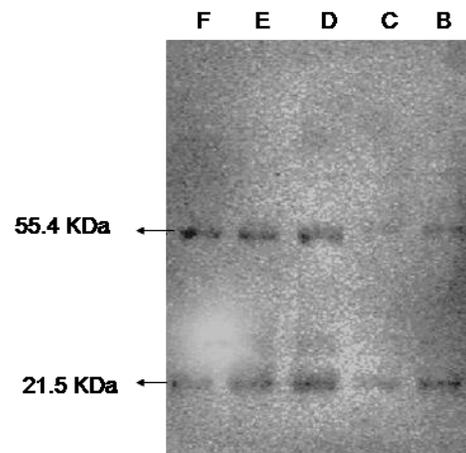


Figure 2: Western Immuno Blot analysis of GAGs associated proteins. Lanes B & C: Unaffected group (Controls); Lanes D, E and F represent GAGs associated (DMB Dye precipitates) proteins of HD Type I, II & III respectively. Western blots were probed with primary anti-sera against aggrecans. Note the presence of intense immuno reactivity with anti aggrecan antibodies for proteins corresponding to MW of 55.4 KDa and 21.5 KDa respectively in HD affected subjects (lanes D, E & F) as compared to unaffected subjects lanes B & C). Conspicuous is the inability of human anti-aggrecan antibodies to react with proteins corresponding to 64.2 and 158.3 KDa MW.

Discussion

A prominent feature in joint disease such as rheumatoid arthritis and osteoarthritis is a loss of aggrecan. The two major cleavage sites are present in the G1 and G2 domains and cleaved by aggrecanases. The lethal chicken mutation of nanomelia shows a severely defective skeletal phenotype in which the extracellular aggrecan is deficient. Nanomelic chondrocytes produce truncated aggrecan molecules that lack the G3 domain and are not secreted from the cell. The truncated aggrecan undergoes xylosylation and GAG chain elongation but is not translocated from Golgi to ER (Li et al. 1993, O'Donnell 1988, Vertel et al. 1994). A similar phenotype is seen in the cartilage matrix deficiency (CMD) mouse, which expresses normal levels of the cartilage-specific collagen type II but fails to express aggrecan (Kimata et al. 1981). The mutation is due to a 7bp deletion in the G1 domain causing a premature stop codon (Watanabe et al. 1994). A recently described mutation in the human causes a truncation of the aggrecan molecule. The affected individuals show a very short stature but with maintained features. This would indicate an

important role of aggrecan in growth cartilage is to expand the matrix. In the brachymorphic mouse, defective sulphation of aggrecan results in mice with a shortened, irregular growth plate. These mice present a phenotype that is characterized by shortened limbs and a domed skull [Orkin et al. 1977]. The defect appears to lie in the channeling mechanism of Adenosine phospho sulphate (APS) between the ATP sulphurylase and APS kinase in the sulphation of the GAG chain, thus resulting in a decrease in the efficiency of the sulphate donor, Phospho Adenosine phospho sulphate PAPS (Schwartz et al. 1998).

The fragments of aggrecan and several of its epitopes that are released in increased amounts in pathological conditions enable measurements to be made in synovial fluid (Mansson et al. 1995). Earlier the DMB dye has been used to analyze urinary glycosaminoglycans to diagnose mucopolysaccharidosis (Whitley et al. 1989; Whitley et al. 2002). In the present context DMB dye had been used to analyze serum GAGs associated proteins.

Table 1: Comparison of densitometric measurements of GAGs associated proteins between Unaffected (Lanes B & C) and HD Affected (Type-I: Lane D, Type-II: Lane E and Type-III: Lane F). Note the less densitometric measurements for 55.4 KDa and 21.5 KDa GAGs associated proteins in unaffected group as compared to HD affected group in SDS- PAGE profile as well as in Western immunoblot profile.

GAGs associated protein	Lane B N=4	Lane C N=4	Lane D N=4	Lane E N=4	Lane F N=4
SDS-PAGE Pattern					
55.4 KDa	21.20± 0.20	22.81± 0.22	39.55± 0.40	40.98± 0.38	35.30± 0.32
21.5 KDa	09.82± 0.10	08.91± 0.10	42.16± 0.41	45.37± 0.42	47.10± 0.42
Western immunoblot pattern					
55.4 KDa	8.92± 0.10	8.24± 0.10	17.21± 0.15	18.39± 0.19	15.05± 0.18
21.5 KDa	5.62± 0.12	4.46± 0.10	20.33± 0.18	16.42± 0.17	15.40± 0.16

Conclusion

The presence of high density of two unknown GAGs associated proteins 21.5 KDa and 55.4 KDa in the serum of HD patients as compared to normal, implies the defective catabolism or anabolism of aggrecan molecule. There may be defective posttranslational modification of aggrecan or loss of aggrecan in HD. These findings may provide insight into the degradation of aggrecan molecule in arthritis and other bone disorders. The limitation of study includes, the sequence of two immunoreactive proteins for anti-human aggrecan antibody. The study also needs to be extended and to be applied for other types of dysplastic and arthritic diseases. The combined effect of Hypocalcemia, Hypocalciuria, Hypocalcitoninemia, Defect in Vit D transduction (Badadani et al. 2010), Prolyl hydroxylase activity (Badadani et al. 2009) and defective aggrecan metabolism with varied degrees may cause this rare disorder with different symptoms (phenotypes).

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