Programs of Excellence in Glycosciences

CLEVELAND CLINIC | Hyaluronan Matrices in Vascular Pathologies

INTER-ALPHA-INHIBITOR ANTIBODY

***PLEASE ACKNOWLEDGE NHLBI AWARD NUMBER PO1HL107147 WHEN YOU PUBLISH RESULTS USING THIS ANTIBODY**

1.1 AN OVERVIEW OF THE ANTIGEN: Since its first description from cultured fibroblasts 15 years ago as a serum derived HA associated protein (SHAP) (1), this unique protein modification of HA has been well characterized as the covalent association of heavy chains (HCs) from **inter-α-inhibitor (IαI)** with HA (Fig. 1) (2). IαI is a serum proteoglycan, synthesized by

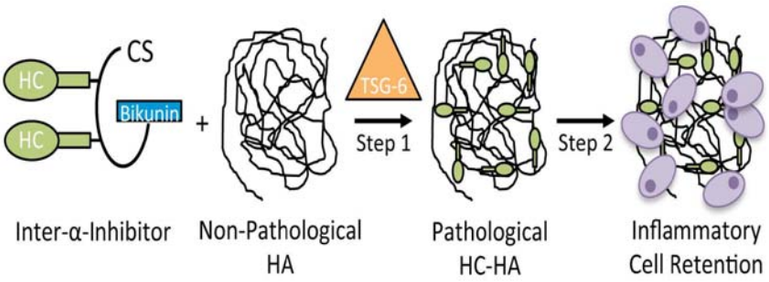

Fig. 1: The Pathological Heavy Chain Modification of Hyaluronan.

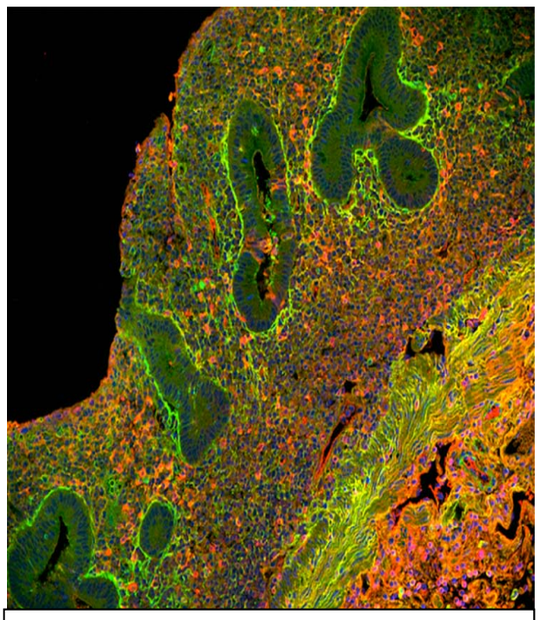

Fig. 2: Heavy Chain – Hyaluronan Staining of an Inflamed Colon. Human paraffin section stained with HABP (green), the Dako IαI antibody (red) and DAPI (blue). (11)

It is composed of 3 polypeptides: the trypsin inhibitor called "bikunin," (35 kDa) and two HCs (~83 kDa each) (4). The two HCs (HC1 and HC2) are covalently attached to bikunin's single chondroitin sulfate (CS) chain by an ester linkage between a HC aspartate and the 6-OH of galNAc on CS. Tumor Necrosis Factor Stimulated Gene 6 (TSG-6) is a 35 kDa protein that is synthesized and secreted by many types of cells after treatment with tumor necrosis factor α (TNFα) and interleukin 1 (5). TSG-6 was identified as the enzyme responsible for the covalent transfer of HCs to HA (6-8) (Fig. 1). The formation of the HC-HA complex has been shown to promote leukocyte adhesion to HA matrices (11-13) and to stabilize HA matrices in cumulus cell – oocyte matrix where it is required for female fertility (6,7,9).

Please direct questions to Ron Midura (midurar@ccf.org; 216-445-3212).

1.2 AN OVERVIEW OF THE ANTIBODY: The IαI antibody from Dako (A0301) is a rabbit polyclonal antibody raised against IαI isolated from human serum. It has been used in numerous studies over the past decade, and is especially recognized for its high titer and sensitivity. By Western, it detects human and bovine bikunin and the HCs of human and mouse IαI (Fig. 3) (10). It is useful for colocalization studies of HCs with HA in paraffin embedded human tissues (Fig. 2) (11). It has been used as a reagent in ELISAs to detect low levels of the HC-HA complex in serum from patients with chronic liver disease and rheumatoid arthritis (13,15,16). It is also useful to measure the HC gel shift from HC-HA to free HCs from tissue extracts treated with *Streptomyces* hyaluronidase (14) (see protocol in 1.4).

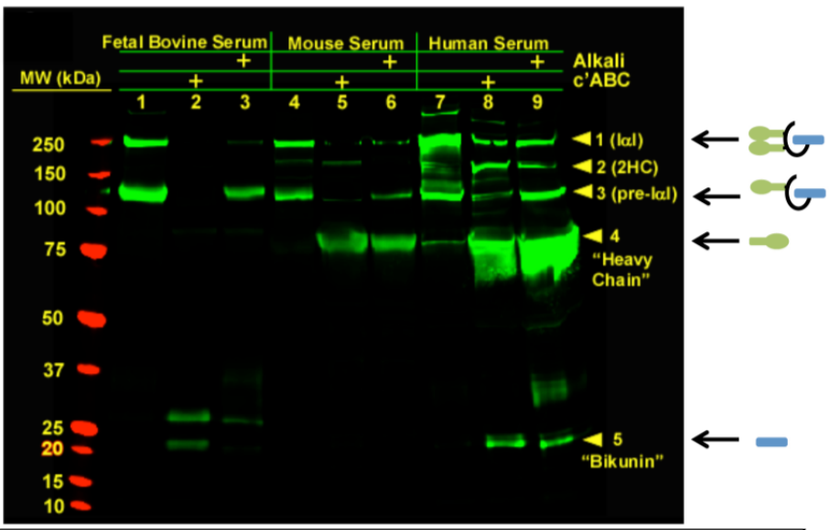

Fig. 3: Species-Specific Sera Reactivity of Dako Inter-Alpha-Trypsin Inhibitor Antibody (A0301). Aliquots of fetal bovine serum (lanes 1-3), mouse serum (lanes 4-6) and human serum (lanes 7-9) were subjected to chondroitinase (c'ABC) or mild alkaline (0.1 M NaOH at RT for 10 min) treatment and analyzed by Western blot, probing the blot with the Dako IαI. This experiment gave identical results when compared with 2 different lots of fetal bovine serum and human serum (not shown). (10)

1.3 RECOMMENDED STORAGE, DILUTIONS AND APPLICATIONS

Solvent: PBS with 15 mmol/L NaN_3

Storage: The antibody is stable for several years at 4° C and is resistant to freeze-thaw.

Western Blot: Recommended Dilution: 1:8000 Species Specificity: Human and Mouse

Immunohistochemistry: Recommended Dilution 1:100 Species Specificity: Human Frozen and Paraffin Sections

1.4 PROTOCOL TO MEASURE THE HEAVY CHAIN MODIFICATION OF HYALURONAN IN TISSUES

This protocol has been described before (14). It describes a method to measure the amount of the Heavy Chain – Hyaluronan complex in tissues.

You will need: *Streptomyces* Hyaluronidase (Seikagaku, 100741-1A or Calbiochem/EMD/Millipore, 389561) Add 500 µl 100 mM ammonium acetate to one ampule of *Streptomyces* hyaluronidase containing 100 TRU of enzyme. Incubate at RT 20 min. Rotate the ampule to make sure that the liquid comes into contact with the entire surface of the bottom quarter of the ampule. Make 25 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 0.2 TRU/µl.

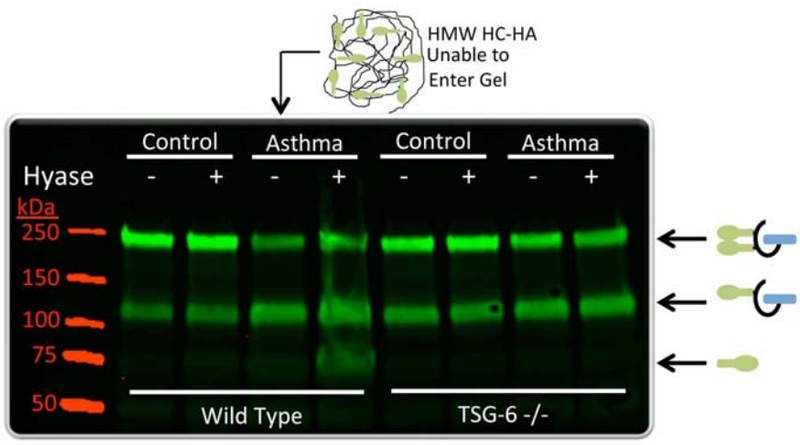

Fig. 4: The Heavy Chain – Hyaluronan Complex Is Present In The Lungs of Asthmatic Wild-Type Mice But Not in TSG-6 -/- Mice. Minced lung tissue from control (saline) or asthmatic (ovalbumin) mice were treated with *Streptomyces* hyaluronidase (Hyase +) or left untreated (-) to release heavy chains from HCHA that is too large to enter the gel. The supernatents of the extracts were analyzed by Western blot and probed with the Dako IαI antibody (green). HCs were released by hyaluronidase treatment from the lungs of asthmatic WT mice but not TSG-6 -/- mice. MW standards are shown in red. Identical results were obtained from three replicates (not shown). (14)

1. **Preparation of the Tissue: (a)** Using a scalpel, cut a small piece of frozen tissue and weigh it on a tared 1.5 ml tube on a sensitive balance. You will need approximately 25 mg of tissue (wet weight). Make sure that the weights from all tissues in your experiment are +/- 1 mg. **(b)** Add 100 µl of pre-chilled PBS for each 25 mg of tissue. **(c)** Finely mince the tissue in the tube on ice using dissecting scissors or a small pestle (such as an *Argos* Pestle Mixer). **(d)** Transfer 50 µl to two new pre-chilled 1.5 ml tubes.
2. **Hyaluronidase Extraction: (a)** Add 10 µl of *Streptomyces* hyaluronidase to one half of each minced tissue sample (50 µl) and add 10 µl of PBS to the other half of sample (50 µl) as a negative control. **(b)** Mix the hyaluronidase by pipetting up/down with a p200 tip with its end cut off to make a wider orifice. **(c)** Incubate on ice for 30 min. **(d)** Centrifuge at 13,000 g at 4° C for 10 min. **(e)** Transfer the supernatent to a new 1.5 ml tube. **(f)** Incubate at 37° C for 30 min. **(g)** Store at -20° C.
3. **Western Blot:** Prepare the samples for Western blot, taking care to load the +/-hyaluronidase side-by-side in adjacent lanes on the same gel (if practical). Probe the blot with the Dako Inter-Alpha-Inhibitor Antibody at a 1:1000 dilution.
4. **Expected Results:** If your tissues have the HC-HA complex, you should see the appearance of the HC band at about 83 kDa on the gel in the + hyaluronidase samples, but not in the – hyaluronidase samples (Fig. 4). Positive controls of HC-HA can be 1 µl/lane of synovial fluid from rheumatoid arthritis patients (17) or by artificially making HC-HA using HMW HA, serum, and recombinant TSG-6 (17).

1.5 REFERENCES

1. Huang L, Yoneda M, Kimata K. A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. J Biol Chem. 1993;268(35):26725-30.
2. Zhao M, Yoneda M, Ohashi Y, Kurono S, Iwata H, Ohnuki Y, et al. Evidence for the covalent binding of SHAP, heavy chains of inter-alpha-trypsin inhibitor, to hyaluronan. J Biol Chem. 1995;270(44):26657-63.
3. Fries E, Kaczmarczyk A. Inter-alpha-inhibitor, hyaluronan and inflammation. Acta Biochim Pol. 2003;50(3):735-42.
4. Milner CM, Tongsoongnoen W, Rugg MS, Day AJ. The molecular basis of inter-alpha-inhibitor heavy chain transfer on to hyaluronan. Biochem Soc Trans. 2007;35(Pt 4):672-6.
5. Wisniewski HG, Vilcek J. Cytokine-induced gene expression at the crossroads of innate immunity, inflammation and fertility: TSG-6 and PTX3/TSG-14. Cytokine Growth Factor Rev. 2004;15(2-3):129-46.
6. Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, et al. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. Development. 2003;130(10):2253-61.

7. Mukhopadhyay D, Asari A, Rugg MS, Day AJ, Fulop C. Specificity of the tumor necrosis factor-induced protein 6-mediated heavy chain transfer from inter-alpha-trypsin inhibitor to hyaluronan: implications for the assembly of the cumulus extracellular matrix. J Biol Chem. 2004;279(12):11119-28.
8. Jessen TE, Odum L. Role of tumour necrosis factor stimulated gene 6 (TSG-6) in the coupling of interalpha-trypsin inhibitor to hyaluronan in human follicular fluid. Reproduction. 2003;125(1):27-31.
9. Mukhopadhyay D, Hascall VC, Day AJ, Salustri A, Fulop C. Two distinct populations of tumor necrosis factor-stimulated gene-6 protein in the extracellular matrix of expanded mouse cumulus cell-oocyte complexes. Arch Biochem Biophys. 2001;394(2):173-81.
10. Lauer ME, Fulop C, Mukhopadhyay D, Comhair S, Erzurum SC, Hascall VC. Airway smooth muscle cells synthesize hyaluronan cable structures independent of inter-alpha-inhibitor heavy chain attachment. J Biol Chem. 2009;284(8):5313-23.
11. de la Motte CA, Hascall VC, Drazba J, Bandyopadhyay SK, Strong SA. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. Am J Pathol. 2003;163(1):121-33.
12. Lauer ME, Cheng G, Swaidani S, Aronica MA, Weigel PH, Hascall VC. Tumor necrosis factor-stimulated gene-6 (TSG-6) amplifies hyaluronan synthesis by airway smooth muscle cells. J BiolChem 2013, Jan 4;288(1):423-31.
13. Zhuo L, Kanamori A, Kannagi R, Itano N, Wu J, Hamaguchi M, et al. SHAP potentiates the CD44mediated leukocyte adhesion to the hyaluronan substratum. J Biol Chem. 2006;281(29):20303-14.
14. Swaidani S, Cheng G, Lauer ME, Sharma M, Mikecz K, Hascall VC, Aronica MA. TSG-6 protein is crucial for the development of pulmonary hyaluronan deposition, eosinophilia, and airway hyperresponsiveness in a murine model of asthma. J BiolChem 2013, Jan 1;288(1):412-22.
15. Shen L, Zhuo L, Okumura A, Ishikawa T, Miyachi M, Owa Y, et al. The SHAP-hyaluronan complex in serum from patients with chronic liver diseases caused by hepatitis virus infection. Hepatol Res. 2006;34(3):178-86.
16. Kida D, Yoneda M, Miyaura S, Ishimaru T, Yoshida Y, Ito T, et al. The SHAP-HA complex in sera from patients with rheumatoid arthritis and osteoarthritis. J Rheumatol. 1999;26(6):1230-8.
17. Lauer ME, Glant TT, Mikecz K, Deangelis PL, Haller FM, Husni ME, et al. Irreversible heavy chain transfer to hyaluronan oligosaccharides by tumor necrosis factor stimulated gene-6. J Biol Chem 2012, Nov 11.