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Analysis of the Heavy Chain Modification and TSG-6 Activity in Pathological Hyaluronan Matrices

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Abstract

During inflammation and developmental processes, heavy chains (HCs) from inter- α -inhibitor (I α I) are covalently transferred to hyaluronan (HA) via the enzyme tumor-necrosis-factor-stimulated-gene 6 (TSG-6) to form a HC-HA complex. In this manuscript, we describe a gel-based assay to detect HC-HA and TSG-6 activity in tissues.

Keywords

Hyaluronan; TSG-6; Heavy chain; Inter- α -inhibitor

1 Introduction

Under normal conditions, hyaluronan (HA) is synthesized as a large (>1,500 kDa) glycosaminoglycan, lacking protein modifications of any kind. During inflammation, a pathological change occurs to HA in which the enzyme, tumor-necrosis-factor-stimulated-gene 6 (TSG-6), transfers heavy chains (HCs) from the serum-derived proteoglycan inter- α -inhibitor (I α I) to the 6-OH of N-acetylglucosamine residues in HA to form a covalent HC-HA complex [1, 2]. While co-localization of biotinylated HA-binding protein (HABP) with HC or I α I antibodies in pathological HA matrices, using fluorescent microscopy, implies the presence of HCs on HA, it does not rule out the possibility that intact I α I is simply bathing the tissue from serum exudates (*see Note 1*). Furthermore, measurement of TSG-6 protein levels from diseased tissues has been difficult due to the low expression levels of this enzyme. Thus, a method to detect TSG-6 activity is likely to improve the sensitivity of TSG-6 detection since a single TSG-6 molecule can produce many measurable HC-HA products. We now describe a gel-based method to document the presence of HC-HA, and to measure TSG-6 activity, in a variety of tissues (*see Note 2*). The principle behind the detection of HC-HA in tissues is that HC-HA is too large to enter a standard SDS-PAGE

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¹We have tested HC antibodies by several other manufacturers and individuals and found that only the Dako I α I antibody has the ability to detect HCs in this assay. Our data suggest that the Dako antibody has an unusually high titer and sensitivity compared to other antibodies. The Dako antibody can be used for both human and mouse tissues.

gel. Upon digestion with a hyaluronidase that specifically digests only HA (*Streptomyces* hyaluronidase), HCs are released from HC-HA, permitting them to migrate as a single ~85 kDa band that can be detected with an antibody against IαI. The principle behind the detection of TSG-6 activity in tissues is that when TSG-6 transfers HCs to a small HA oligosaccharide (HA14 for example), a single HC band appears on an SDS-PAGE gel at ~85 kDa plus the MW of the oligo. Thus, in the presence of endogenous IαI and an exogenous HA oligo added to the reaction mixture, TSG-6 activity can be detected by the appearance of a HC band on a Western blot probed with an antibody against IαI (Fig. 1). The reader is also advised to consult a recently published, alternate, ELISA based method for measuring TSG-6 activity [3].

2 Materials

1. Approximately 75 mg (wet weight) of frozen tissue is needed for this assay (*see Note 3*).
2. *Streptomyces* hyaluronidase (EMD Millipore; 389561): Add 0.5 ml of PBS to one vial containing 100 U of enzyme. Gently swirl and incubate at RT for 10 min. Freeze 25 µl aliquots at −80 °C.
3. Any hyaluronan oligosaccharide containing 8–20 monosaccharides can be used. Oligosaccharides containing 8 and 10 monosaccharides can be purchased from Hyalose, LLC (HYAOLIOG8EF-1 and HYA-OLIGO10EF-1, respectively). Solubilize the oligosaccharide at 1 mg/ml in water.
4. Recombinant TSG-6 (R&D Systems; 2104-TS-050): Add 200 µl PBS to 50 µg of the dried protein to make a 5 µg/ml stock solution, which can be stored at −80 °C.
5. Polyclonal IαI antibody (*see Note 1*) (A0301, Dako North America, Inc. Carpinteria, CA): This antibody is also available through the Program of Excellence in Glycosciences (<http://pegnac.sdsc.edu/cleveland-clinic/files/PEGNAC-DAKO-Inter-alpha-Inhibitor-Antibody-v1-1.pdf>).
6. SDS-PAGE gels (4–15 % acrylamide) and appropriate reagents and equipment for Western blotting.
7. Additional miscellaneous items: Analytical balance (1 mg sensitivity), dry ice, forceps, No. 15 scalpel, eye protection, fine scissors, 1.5 ml tube Pestle Mixer, 10 cm Petri dishes.

²We have found that it is difficult to process more than four samples at a time in this assay. The individual mincing of each tissue, followed by relatively short incubation times (i.e., 30 min), makes it difficult to process more than four samples while maintaining the schedule outlined in this protocol. Deviation from the suggested incubation times may result in lower sensitivity (for shorter incubation times) or proteolytic degradation of HCs (for longer incubation times). If more than four samples need to be processed, we recommend processing them in separate batches.

³It is important not to let the tissue thaw until ready to begin the mincing step (**step 6**). The tissues should be kept on dry ice until this step to prevent proteolytic degradation. We recommend pre-chilling the tubes to the temperature described in each step to keep the temperature constant from step to step.

3 Methods

1. Label 1.5 ml centrifuge tubes. Weigh the tubes and record their weights to within 1 mg in a lab notebook. Chill on dry ice. Leave them on dry ice unless otherwise instructed. It is essential to keep the tissue frozen until it is minced (**step 6**).
2. Transfer frozen tissue to dry ice and bring it to a lab bench. Using forceps, transfer a tissue chunk to a 10 cm Petri dish pre-chilled on dry ice. With appropriate eye protection, use a No. 15 scalpel to cut off a chunk of tissue approximately the volume of 100 μ l. Transfer the cut tissue into a pre-weighed 1.5 ml tube.
3. Repeat **step 2** for all of the other tissues.
4. Weigh the tubes, containing the cut tissues, and record the weights to within 1 mg in a lab notebook. Note that 75 mg of tissue is the minimum amount of tissue needed for this assay. Calculate the amount of pre-chilled PBS to add to each tissue such that there is 0.33 mg tissue for each 1 μ l of PBS.
5. Label four new tubes for each tissue (tube #s described below).
6. Mince one of the tissues in the following manner: Transfer one of the tissues from dry ice to wet ice and let it thaw. Keep the tissue on wet ice during the mincing procedure. Using a pair of fine scissors, cut the tissue into pieces as small as possible (about 1 min, *see Note 4*). If available, we recommend the use of a bead homogenizer instead of scissors, such as the Beadbug Microtube Homogenizer (Benchmark Scientific).
7. Cut the tip off of a 200 μ l pipet tip to widen the orifice. Transfer 50 μ l aliquots of the minced tissue from **step 6** into the four new tubes labeled in **step 5**. Keep these on wet ice. Treat them as follows:
 - (a) Tube #1 (the “– hyaluronidase” control): Add 5 μ l of PBS to 50 μ l of minced tissue and incubate on ice for 30 min. Centrifuge at $13,000 \times g$ for 5 min at 4 °C and transfer the supernatant to a new tube. Incubate the supernatant at 37 °C for 30 min. After the 30-min incubation, store the supernatant in the freezer until ready to analyze by Western blot. Discard the pellet.
 - (b) Tube #2 (the “+ hyaluronidase” control): Add 5 μ l of Streptomyces hyaluronidase (at 100 TRU per 500 μ l) to the 50 μ l extract and incubate on ice for 30 min. Centrifuge at $13,000 \times g$ for 5 min at 4 °C and transfer the supernatant to a new tube. Incubate the supernatant at 37 °C for 30 min. After the 30-min incubation, store the supernatant in the freezer until ready to analyze by Western blot. Discard the pellet.
 - (c) Tube #3 (measurement of endogenous TSG-6 activity): If the sample does not contain serum, or it is expected to have low levels of serum, add

⁴Effective mincing of the tissue is essential to the highest yield possible for each sample. Mincing of different types of tissues may require more or less mincing time for effective mincing. Once mincing with the scissors, or homogenizer, no longer has any further effect, additional mincing should be stopped.

2 μ l of human serum to each 50 μ l of human serum to each. Add 2 μ l of an HA oligosaccharide (from HA 8-14) (stock concentration at 1 mg/ml) and 3 μ l of PBS to 50 μ l of minced tissue and incubate at 37 °C for 2 h. Centrifuge at $13,000 \times g$ for 5 min at 4 °C, transfer the supernatant to a new tube, and store the supernatant in the freezer until ready to analyze by Western blot. Discard the pellet.

- (d) Tube #4 (positive control for TSG-6 activity): If the sample does not contain serum, or it is expected to have low levels of serum, add 2 μ l of human serum to each 50 μ l aliquot of sample to provide a heavy chain donor (i.e. inter-alpha-inhibitor). Add 2 μ l of an HA oligosaccharide (from HA 8-14) (stock concentration at 1 mg/ml), and 2 μ l of recombinant TSG-6, and 1 μ l of PBS to 50 μ l of minced tissue and incubate at 37 °C for 2 h. Centrifuge at $13,000 \times g$ for 5 min at 4 °C, transfer the supernatant to a new tube, and store the supernatant in the freezer until ready to analyze by Western blot. Discard the pellet (*see Notes 5 and 6*).

8. Repeat **steps 6 and 7** for the remaining tissues.

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⁵We have noticed that endogenous TSG-6 activity is rarely detectable in end-stage diseased tissues in which inflammation has reached a chronic and advanced state. For example, the colon tissue analyzed in this manuscript came from a portion of colon that was surgically removed from a patient with ulcerative colitis because of the advanced stage of disease in that region. Furthermore, the lung tissue presented in this manuscript was postmortem tissue that came from a patient who died of asthma. Although we are unable, at this time, to publish existing data demonstrating endogenous TSG-6 activity, we are able to propose that tissues from less advanced stages of disease may demonstrate endogenous TSG-6 activity.

⁶The manuscript by Swaidani et al. [4] was the first to utilize the gel-based method of HC-HA detection as outlined in this protocol. It demonstrated that HC-HA was not detectable in the lungs of naïve wild-type or TSG-6^{-/-} asthmatic mice, but was readily present in wild-type asthmatic mice. This emphasizes the importance of TSG-6 in the formation of HC-HA and shows that, at least in the airways, TSG-6 is essential for the formation of HC-HA.

Fig. 1. Western blot demonstrating the presence of the pathological form of hyaluronan (i.e., HC-HA) in inflamed colon and lung tissue from human patients with ulcerative colitis and asthma (panels **a** and **b**, respectively). This blot was probed with the Dako IaI antibody (green). Molecular weight standards are shown in red. The green lobules in the cartoon models represent HCs attached to bikunin (blue rectangle) via a single chondroitin sulfate chain (black line). The cartoon with two HCs represents IaI. The cartoon with one HC represents pre-IaI. When hyaluronidase (HAase) was added to the minced tissue, the HC-HA complex, which was too large to enter the gel, was digested, releasing HCs that migrate as a single band at ~85 kDa (compare lanes 1 and 2 for the gut tissue, and lanes 5 and 6 for the lung tissue). When an HA oligo, 14 monosaccharides in length, is added to the minced tissue, endogenous TSG-6 activity has an HA substrate on which to transfer HCs from endogenous IaI, which now appear as a single ~85 kDa band on the gel (lanes 3 and 7). Recombinant TSG-6 was added as a positive control for TSG-6 activity (lanes 4 and 8), proving that the reaction conditions were suitable for measuring TSG-6 activity if endogenous TSG-6 was present. In both the colon and lung tissues, no detectable endogenous TSG-6 activity was present. The broader HC band toward lower molecular weights in the colon tissue (lanes 2 and 4) may reflect endogenous protease activity in the original tissue. Furthermore, the smearing of the IaI band in the colon tissue (lanes 3 and 4) could also be caused by endogenous proteolytic activity during the 2-h incubation at 37 °C. Notice that this smearing was not observed in the lung tissue (lanes 7 and 8) which was treated the same as the colon tissue