A Modified ELISA Accurately Measures Secretion of High Molecular Weight Hyaluronan (HA) by Graves’ Disease Orbital Cells

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Excess production of hyaluronan (hyaluronic acid [HA]) in the retro-orbital space is a major component of Graves’ ophthalmopathy, and regulation of HA production by orbital cells is a major research area. In most previous studies, HA was measured by ELISAs that used HA-binding proteins for detection and rooster comb HA as standards. We show that the binding efficiency of HA-binding protein in the ELISA is a function of HA polymer size. Using gel electrophoresis, we show that HA secreted from orbital cells is primarily comprised of polymers more than 500,000. We modified a commercially available ELISA by using 1 million molecular weight HA as standard to accurately measure HA of this size. We demonstrated that IL-1β-stimulated HA secretion is at least 2-fold greater than previously reported, and activation of the TSH receptor by an activating antibody M22 from a patient with Graves’ disease led to more than 3-fold increase in HA production in both fibroblasts/preadipocytes and adipocytes. These effects were not consistently detected with the commercial ELISA using rooster comb HA as standard and suggest that fibroblasts/preadipocytes may play a more prominent role in HA remodeling in Graves’ ophthalmopathy than previously appreciated. (Endocrinology 155: 627–634, 2014)

Graves’ ophthalmopathy (GO) is an autoimmune disorder in which the pathogenesis appears to involve binding of autoantibodies to TSH receptors (TSHRs) on cells in the retro-orbital space leading to tissue remodeling that may lead to optic nerve compression (1). According to histologic studies, retro-orbital tissue from GO patients is characterized by interstitial edema and hyaluronan (hyaluronic acid [HA]) accumulation (2), which is the likely source of tissue swelling and proptosis, and appears to be responsible for the major signs and symptoms in severe GO.

The mechanism through which Graves’ autoantibodies (Graves’ disease [GD]-IgG) initiate remodeling in such a specific, nonthyroid tissue is still a subject of debate. In the current model for GO pathogenesis, GD-IgGs are suggested to target fibroblasts in the retro-orbital tissue (1). Although several ideas exist for what happens after antibody binding, the most straightforward hypothesis is that autoantibody binding to TSHR on orbital fibroblasts induces differentiation into adipocytes (adipogenesis), which causes adipose tissue expansion, increased TSHR expression, and HA production. Orbital fibroblasts from patients with GO (GOFs) in culture are capable of undergoing differentiation into adipocytes when cultured in certain mediums and are therefore considered preadipocytes. TSHR stimulation with activating antibodies or TSH has been shown to up-regulate adipogenic markers in GOFs (3) and other cell types (4, 5). After differentiation, GOFs demonstrate greater stimulation of HA production as well as increased expression of HA synthase (HAS) genes upon TSHR activation (6–8). However, increased HA produc-
tion could come from fibroblasts/preadipocytes or adipocytes, or both.

Difficulties in delineating GO pathogenesis stem from the fact that TSHR stimulation of undifferentiated GOFs in vitro does not produce a consistent HA response. Studies using commercial ELISAs to measure HA production showed only small changes in HA secretion from undifferentiated compared with adipocyte-differentiated GOFs (Adipo-GOFs) (9, 10). Pharmacologic stimulation of TSHR signaling pathways was shown to increase HA yield and HAS expression in undifferentiated GOFs but not with TSH treatment (10, 11). Transfection of constitutively active TSHR into GOFs led to significant increases in HA secretion and up-regulation of HAS isoforms, but in these experiments, signaling pathways were artificially robustly induced (7). Under certain conditions, the GD-IgG activating monoclonal antibody from a patient with GD (M22) and IL-1β both moderately increased HA in undifferentiated GOFs. However, changes were 2-fold at most (12, 13). In contrast, studies that measured HA synthesis using radiolabeled precursor incorporation detected higher-fold increases in HA production in response to IL-1β (14, 15). Although HA levels measured in ELISAs are the result of a steady state between synthesis and degradation, the difference in measurements between the 2 methods suggests that the commercially available ELISAs may underestimate HA production.

The physiochemical properties of HA greatly differ from most factors usually studied in ELISAs. HA is a linear, highly anionic, nonsulfated polysaccharide found in the extracellular matrix. HA exists in different lengths, depending on its tissue source and disease state. HA from amniotic membrane extracts has an average molecular weight of 3 million (16), whereas HA from rooster comb is reported to range from 800 to 1000 (17–19). In vitro, cultured synovial membranes produce polydispersable HA mixtures ranging in size from 5000 to more than 3 million (20, 21), and HA polymers secreted from myofibroblasts are usually more than 500 000 (22, 23). However, the most commonly applied, commercially available ELISAs (24) used to measure HA concentration do not take HA size into account nor consider whether differences in size profiles between samples have any effect on concentration measurements. In these assays, HA concentration is dependent on binding to an HA-binding protein (HABP).

The efficiency of binding of HAs of various polymer sizes to HABP, however, has not been considered in the GO field. Previous experiments, in which radiolabeled precursor incorporation into HA was measured, differences in size are not taken into account when determining synthetic rate. Thus, determining the size distribution of HA secreted by orbital fibroblasts is not only important in interpreting HA assays but also may be an overlooked aspect in GO pathogenesis.

Using an HABP-independent method to measure HA from GOFs in vitro, we show that HA secreted from GOFs has a markedly different size distribution than the rooster comb HA used as standards in commercial ELISAs. Furthermore, HA polymer size affects its binding to HABP. Because of these differences, HA ELISAs underestimate changes in HA secretion from GOFs. Based on data from our HABP-independent studies, we used an HA ELISA with a 1 million molecular weight HA standard that accurately measures HA concentrations in our system and demonstrated a greater effect of IL-1β that was unique to GOF preadipocytes. Most importantly, we showed that TSHR stimulation by both TSH and monoclonal-activating antibody M22 increases HA secretion in undifferentiated GOFs. These changes were obscured using rooster comb HA standard provided in commercial ELISAs and suggest that the importance of TSHR signaling pathways in preadipocyte GOFs has been undervalued in the pathogenesis of GO.

Materials and Methods

Primary orbital fibroblast cell culture

Primary orbital fibroblasts from Graves’ and non-Graves’ patients were generously provided by Rebecca Bahn (Mayo Clinic, Rochester, Minnesota) and harvested as previously described (8). Fibroblast strains were obtained from 4 Graves’ patients undergoing orbital decompression surgery (GOFs) and 2 patients with no history of Graves’ disease (normal orbital fibroblasts, NOFs). Use of these samples was approved by the Mayo Clinic Institutional Review Board, and studies were carried out according to the Institutional Review Board guidelines. Thawed cells were propagated in growth medium (DMEM, 4.0 mM L-glutamine and sodium pyruvate, 10% fetal bovine serum (FBS) and 1% penicillin/gentamicin solution), in a humidified 10% CO2 incubator at 37°C. Differentiation was induced in confluent fibroblast cultures with adipogenic medium (DMEM, 4.0 mM L-glutamine and sodium pyruvate, 10% FBS, 1% penicillin/gentamicin solution, 0.1 mM indomethacin, 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin). Adipogenesis was confirmed as the level of adiponectin increased more than 10-fold (25). For experiments, cell passage was no greater than passage 10.

HA induction

GOFs and NOFs were grown to confluence in 12-well plates in growth medium until proliferation was arrested through contact inhibition, verified visually by phase-contrast microscopy when less than 1% of cells were seen dividing. This ensured an equal number of cells per well during the course of the experiment. Cells were treated with growth medium without or with IL-1β (10 ng/mL; Sigma), activating TSHR antibody M22 (100 ng/mL; Kronos), or bovine TSH (100 mU/mL; Sigma). Adi-
Adipocytes were pretreated 3 days and then switched to adipogenic medium without or with IL-1β, M22, or TSH and differentiated 7 days. Preadipocytes were treated for a total of 10 days to match the treatment time of adipocytes. Medium was replaced every 3–4 days, and conditioned media were collected, combined, and stored at 4°C overnight or −20°C for long-term storage.

For TSHR inhibition experiments, TSHR antagonist National Institutes of Health Chemical Genomics Center number 00229600 (TSHR-ant) was synthesized by the National Center for Advancing Translational Science, National Institutes of Health as previously reported (26). GOF preadipocytes were pretreated with TSHR-ant (10μM) for 24 hours. Cells were treated with bovine TSH (100 mU/mL; Sigma) or M22 (100 ng/mL) in growth medium without or with TSHR-ant (10μM). Medium was refreshed after 2 days, and total treatment time was 4 days.

For HA time-course experiments, GOFs were grown to confluence in growth medium, then starved for 1 day in low-serum medium containing 1% FBS. Cells were treated with hyaluronidase from *Streptomyces hyalurolyticus* (1 U/mL; Sigma) in Hanks’ balanced salt solution for 1 hour at 37°C to remove existing HA. After several washes with Hanks’ balanced salt solution, cells were switched to low-serum medium with IL-1β (10 ng/mL). Conditioned media were collected and stored at −20°C.

**HA polyacrylamide gel electrophoresis (PAGE)**

Conditioned media were filter concentrated 5 times (Corning Spin-X UF, MWCO 5,000). To enrich for HA, conditioned media were digested with proteinase K (50 μg/mL; Roche) for 4 hours at 55°C followed by boiling for 10 minutes for enzyme inactivation. Negative controls were produced by treating sample aliquots with hyaluronidase for 16 hours at 37°C to remove existing HA. After several washes with Hanks’ balanced salt solution, cells were switched to low-serum medium with IL-1β (10 ng/mL). Conditioned media were collected and stored at −20°C.

**HA/HABP blotting**

HA samples were separated by PAGE as described above. After electrophoresis, gels were soaked in freshly prepared HA depolymerization buffer (0.2mM cupric sulfate and 2mM ascorbic acid in TBE) for 30 minutes at room temperature with shaking, then washed 3 times for 10 minutes in TBE. HA was transferred to nylon membranes in 0.5X TBE at constant 100 mA overnight at 4°C in a Bio-Rad Mini Trans-Blot apparatus. Immediately after transfer, membranes were blocked with Odyssey blocking buffer (LiCor) containing 1% sodium dodecyl sulfate for 1 hour at room temperature. To detect HA, membranes were incubated with 5-ng/mL biotin-HABP (Calbiochem) in Odyssey blocking buffer plus sodium dodecyl sulfate overnight. Blots were washed 2 times with 1× phosphate-buffered saline/Tween 20 (0.1% Tween 20), rinsed with PBS, and then incubated in the dark with diluted IRDye 800CW Streptavidin (1:10,000; LiCor) for 30 minutes. Blots were imaged on a LiCor Odyssey CLX.

**Enzyme-linked immunosorbent assay**

A commercial ELISA was obtained from Corgenix and run according to the manufacturer’s instructions with 1 exception. In addition to the supplied standards that were diluted 1:10, the 500- and 800-ng/mL rooster comb standards were diluted 1:5 to produce 1000- and 1600-ng/mL standards, respectively. Binding of different sized HA (LifeCore) to HABP was measured using the ELISA platform but substituting various HA of the following HA preparations: 20,000 (actual 28,600), 200,000 (actual 215,000), and 1 million (actual 1,01 million). We analyzed rooster comb HA (lot 095K3786; Sigma) and found the size range to be from 50,000 to 500,000. Modified ELISAs for measurement of HA secretion were based on the Corgenix kit, with the rooster comb standard replaced by freshly prepared 1 million molecular weight HA.

**Statistical analyses**

Significance was determined using Student’s t test.

**Results**

**Cultured orbital fibroblasts secrete primarily high molecular weight (HMW) HA**

A modified PAGE technique was used to assess the size distribution of HA from GOFs and NOFs and from these cells after differentiation to adipocytes (Adipo-GOF and Adipo-NOF, respectively).

Previous HA electrophoresis experiments used polyacrylamide gels to resolve low molecular weight (LMW) HA, ranging from 5000 to 500,000, and agarose gels to separate HMW HA, 500,000 and above. Here, 3%–12% NativePAGE polyacrylamide gels were used to visualize both LMW and HMW HA on the same gel. Individual bands, ranging from 50,000 to 500,000 in the Select-HA LoLadder, were resolved, whereas HA from the Select-HA HiLadder migrated as a single band with a mobility similar to that of 500,000 HA (Figure 1A). When visualized with Stains-All, the amount of HA polymers greater than 500,000 can be analyzed by the intensity in the single band, because the density of the band was found to be proportional to the amount of HA from 125 to 4000 ng (slope = 3.3 ± 0.2 arbitrary units/ng; R² = 0.98).

HA was purified from conditioned media collected from orbital fibroblasts, and the size distribution of cell-secreted HA was compared with HA from rooster comb extracts and purified HA of various sizes. Rooster comb HA was shown to be a polydisperse mixture with a significant proportion of LMW polymers and apparent sizes ranging from 50,000 to 500,000 (Figure 1A). In contrast, HA from orbital fibroblasts migrated as single HMW
bands (Figure 1A) with a similar mobility to purified 1 million molecular weight (MW) HA (Figure 1A, lane 3). Hyaluronidase treatment confirmed that HA was the dominant species in those bands (Figure 1A), and an HA/HABP blot demonstrated binding of HABP to these bands that was abolished by hyaluronidase treatment (Figure 1B).

**HABP preferentially binds HMW HA**

We found that HAs of different sizes bind differently to a fixed amount of HABP as is present in commercial kits (Figure 1C); 20 000 MW HA bound very poorly. Rooster comb HA bound less than the purified 200 000 HA, which bound moderately well. Most importantly, 1 million MW HA, which was most similar in size to cell-secreted HA, bound most efficiently to the HABP. Thus, it is clear that in order to accurately measure HA, a standard for the ELISA must be of a similar size to the predominant HA in the sample, and using rooster comb HA, which is comprised of a broad range of HA sizes that are less than 500 000 MW, would not accurately measure HA secreted by orbital fibroblasts and adipocytes. We, therefore, used a modified ELISA, in which the standard was 1 million MW HA for all our measurements.

**IL-1β regulates HA secretion differently in GOFs and NOFs**

Because IL-1β has been shown to regulate HA secretion in a number of reports (14, 15), we decided to confirm the accuracy and sensitivity of the modified ELISA using undifferentiated GOFs, which secreted less HA than Adipo-GOFs (see below). Because optimal adipogenesis requires cells to be cell cycle-arrested at confluence, to compare cells under similar conditions, we compared HA secretion from undifferentiated and differentiated cells at confluence. HA electrophoresis on 3%–12% NativePAGE gels was used as an HABP-independent assay to determine the effects of IL-1β treatment on HA secretion. According to densitometry results from multiple GOF strains, total HA in the conditioned media was on average 6.7-fold greater than controls after 10 days of continuous IL-1β treatment, which was similar to that measured by the modified ELISA (see below) but was several fold higher than that detected using the commercial ELISA (1.3-fold). PAGE also showed that the size distribution of HA secreted by GOFs equal masses of HAs of different sizes were applied. C, Purified 20 000, 200 000, and 1 million MW HA polymers and rooster comb HA were processed in a commercial HA ELISA. The HA-HABP interaction was determined by the colorimetric signal and demonstrates that, for a given mass of HA, HABP signal is greater for larger polymers. A mixture of lower size HAs, such as rooster comb, results in a signal much lower than the HMW HA seen in fibroblast samples.
under several culture conditions (Figure 1A), including IL-1β, did not change and was predominantly HMW.

In order to make more accurate and sensitive measurements of multiple samples, all further HA measurements were performed using the modified ELISA. Because the HA secreted by orbital fibroblasts under all the culture conditions explored in this study migrated with an apparent size between 500,000 and 1.5 million, we used a 1 million MW HA as standard in the modified ELISA. In contrast to the commercial ELISA, the modified ELISA measured significant increases in HA secretion with IL-1β treatment of GOF preadipocytes after as early as 2 days (Figure 2A). Total HA levels increased at constant rates under both control and IL-1β-stimulated culture conditions, but cells treated with IL-1β secreted HA at a faster rate. Over 6 days, the average rate of HA secretion for the 4 strains was 3.6-fold greater in GOFs treated with IL-1β than control media (Figure 2B) and was even higher when additional GOFs were studied (7.8-fold) (Figure 3).

We next compared the effects of IL-1β on HA secretion by undifferentiated GOFs, Adipo-GOFs, undifferentiated NOFs, and Adipo-NOFs (Figure 3). NOFs secreted HA at a level higher than GOFs but were not affected by IL-1β treatment in contrast to stimulation found with GOFs. Differentiation to adipocytes increased HA secretion by NOFs (3.3-fold) and GOFs (12.8-fold), but IL-1β decreased HA secretion by differentiated NOFs, whereas it had little effect in differentiated GOFs.

**TSHR activation is sufficient for HA induction in GOFs**

TSHR activation by the monoclonal antibody M22 stimulated HA secretion to a much greater extent in GOFs than NOFs (Figure 4A). Undifferentiated NOFs (1.8-fold)
and Adipo-NOFs (1.5-fold) exhibited small increases in HA secretion in response to exposure to TSHR-activating antibody M22. In contrast, M22 stimulated HA secretion by undifferentiated GOFs and Adipo-GOFs by 7.6- and 2.4-fold, respectively. Of note, the fold increase in Adipo-GOFs was from an elevated control level.

We used an antagonist of TSHR activation (25, 26) to determine whether the stimulation of HA secretion caused by M22 was mediated by TSHR, because it has been suggested that M22 may have other receptor targets (10–12, 27). TSH alone stimulated HA secretion in undifferentiated GOFs as soon as 4 days. However, HA levels only reached 50% of what was found with M22 (Figure 4B). The effects of TSH and M22 were completely inhibited by the TSHR antagonist.

Discussion

Preferential binding of HABP to HMW HA

Unlike other chemical reactions involving large macromolecules, an HA polymer is equally reactive along its entire surface, and the kinetic assumptions used to design traditional ELISAs would not apply. In a mixture of HA polymers, overall, HABP signal would be the result of some sort of averaged binding, dominated by the signal from larger polymers. The polymer size and polydispersity of rooster comb HA will vary lot to lot depending on the age, breed, and hormonal state of the rooster and method of extraction. Obtaining these details about the HA supplied in commercial HA ELISA kits is difficult. We found that commercially acquired rooster comb HA contained a significant LMW component despite supplier claims. This rooster comb HA sample likely had a similar polydispersity to the HA ELISA standard, because its concentration measurements were virtually 1:1 with actual concentration. The LMW HA extracted from rooster comb bound less HABP than the same mass unit of HMW HA. Therefore, ELISAs on HA from orbital fibroblasts would follow a pattern akin to 1 million MW HA, where concentration would be overestimated at low concentrations and underestimated at high ones. Analyzing the size distribution of samples and then using the appropriately sized HA as standard revealed major errors in the measurements made using currently available ELISAs with rooster comb HA as standard. These findings led us to reexamine some basic hypotheses in GO pathogenesis.

NOFs differ from GOs in their regulation of HA secretion by IL-1β

Both NOFs and GOs exhibited marked increases in HA secretion when differentiated into adipocytes. One of the unexpected outcomes was the absence of a stimulatory response to IL-1β in undifferentiated NOFs compared with the robust response in undifferentiated GOs. In addition, IL-1β inhibited HA secretion in differentiated NOFs, whereas it had little effect in differentiated GOs. The reason(s) for these differences is not apparent at the present time. The greater effect of IL-1β in GO preadipocytes compared with adipocytes confirm the findings in a previous report (28) that undifferentiated GOs have a robust response to IL-1β. Under all conditions we tested, IL-1β did not change HA size distribution. In our system, HA degradation apparently did not occur, because no LMW HA was observed. Our finding of no HA degradation in GO cultures supports the conclusion of a previous study (29).
TSHR’s role in matrix remodeling may begin before GOF differentiation

We found that the ability of TSHR activation to stimulate HA secretion in undifferentiated GOFs is much greater than previously thought. We cannot distinguish whether HA induction is the result of increased synthase expression/activity or decreased hyaluronidase expression/activity. Both undifferentiated GOFs and NOFs responded to M22, although GOFs responded to a greater degree. Undifferentiated GOFs have been reported to exhibit higher levels of TSHR expression compared with NOFs (8, 30–33) that may account for the amplified effect of M22 in GOFs. Both cell types are expected to up-regulate TSHR expression with adipogenesis (34–36), and this may account for the greater effects of M22 in orbital adipocytes compared with undifferentiated fibroblasts. In some previous studies, TSHR activation in undifferentiated GOFs by TSH was found not to increase HA secretion (10, 12). The authors concluded that TSHR activation was not a primary pathway for HA secretion and that signaling by M22 must occur through a different receptor. Our data do not exclude effects by M22 being mediated by other receptors, but, as we have shown previously (37), they do demonstrate that TSHR activation alone (by TSH) is capable of stimulating HA secretion. Moreover, the findings that the specific TSHR antagonist fully inhibited HA secretion stimulated by TSH and M22 suggest that TSHR is the major target of M22 with regard to HA secretion.

In conclusion, we have shown that measurement of HA levels by assays that use HABP to bind HA are dependent on the size(s) of HA in the sample and in the standards. We suggest that before using an HABP-dependent ELISA, it is necessary to determine the size of the predominant HA polymer in the samples and then to use a similarly sized purified HA as standard. Using this approach, we have been able to accurately and sensitively measure HA levels in the conditioned mediums from orbital fibroblasts and adipocytes in culture. Our confirmation of previous data by Kumar et al (12) that TSHR activation can lead to increased HA secretion from undifferentiated GOFs suggests that fibroblasts and adipocytes in the orbital tissue of patients with Graves’ disease may be responsible for the matrix remodeling found in GO. Moreover, these data are consistent with the idea that matrix remodeling may occur early in the pathogenesis of GO before any increase in adipocyte number.

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References

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