A comparison of the sensitivity, specificity, and molecular weight accuracy of three different commercially available Hyaluronan ELISA-like assays

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Received on June 21, 2010; revised on August 18, 2010; accepted on September 14, 2010

Hyaluronan (HA) is a glycosaminoglycan found in the extracellular matrix and ranges from several thousand to millions of daltons in size. HA has importance in various pathological conditions and is known to be elevated in several diseases. Three commonly used, commercially available HA enzyme-linked immunosorbent assay (ELISA)-like assays (from Corgenix, Echelon and R&D) were compared on the basis of accuracy, sample variability and ability to measure a range of HA sizes. The Corgenix HA ELISA-like assay displayed the lowest intra-assay variability [coefficient of variation (CV) = 11.7 ± 3.6%], followed by R&D (CV = 12.3 ± 4.6%) and Echelon (CV = 18.9 ± 9.2%). Interassay variability was also lowest for the Corgenix assay (CV = 6.0%), intermediate for the Echelon assay (9.5%) and highest for the R&D assay (CV = 34.1%). The high interassay variability seen for the R&D assay may have been due to the effect of dilution, since the dilution-independent interassay variability was 15.5%. The concentration of the standard HA was overestimated by the Echelon assay by 85% and underestimated by the R&D and Corgenix assays by 34 and 32%, respectively. The Echelon HA ELISA-like assay was the most effective at measuring all sizes of HA tested (2 MDa and 132, 66 and 6.4 kDa), whereas the Corgenix and R&D assays were unable to detect 6.4 kDa HA. These findings suggest that the Echelon HA ELISA-like assay is better suited for size-sensitive HA measurements but has a relatively high variability. The Corgenix and R&D HA ELISA-like assays have low variability and high accuracy but are not suitable for detecting low-molecular-weight HA.

Keywords: ELISA/hyaluronan/molecular weight/variability

Introduction

Hyaluronan (HA) is a glycosaminoglycan (GAG), which is found in the extracellular matrix (ECM) and pericellular matrix (Taylor and Gallo 2006). Unlike other GAGs, HA does not have a core protein and is not part of a proteoglycan, although it does have many protein-binding partners (Taylor and Gallo 2006). HA is composed of repeating disaccharide units containing β-D-N-acetylglucosamine and D-glucuronic acid. It is widely distributed in the body and is abundant in connective tissue and in the ECM (Delpech et al. 1997; Evanko and Wight 1999). HA has mechanical functions in the synovial fluid, cartilage and vascular wall, and the HA polymer volume can increase up to 10,000 times through the absorption of water (Csoka et al. 2001). The size of HA can range from several kilodaltons to over 4 MDa (Jiang et al. 2007). It contributes to cellular migration and proliferation, especially in tissue repair. HA is synthesized by a group of three 7-transmembrane HA synthases (HAS; Philipson and Schwartz 1984; Itano et al. 1999) and is broken down by hyaluronidases (Hyal). Although there are five Hyal genes and one Hyal pseudogene in the human genome, Hyal1 and Hyal2 are thought to play the largest role in the breakdown of HA (Csoka et al. 1999). Hyal2 is glycosylphosphatidylinositol-anchored to the cell membrane and is able to break HA down to 20 kDa fragments that are enclosed in endocytic vesicles, transported into the cell and degraded further by Hyal1 (Rai et al. 2001). The size of the HA molecule affects its biological function, with smaller sizes being proangiogenic and proinflammatory and larger sizes homeostatic (West et al. 1985; Termeer et al. 2000; Stern 2006, 2009).

The importance of HA is highlighted by its involvement in a variety of pathological conditions including pulmonary hypertension (PH; Aytekin et al. 2008), liver cirrhosis (Nyberg et al. 1988; Tamaki et al. 1996; Wong et al. 1998; Gressner et al. 2007) and ovarian, colorectal and bladder cancers (Ropponen et al. 1998; Anttila et al. 2000; Auvinen et al. 2000; Lokeshwar and Soloway 2001; Simpson and Lokeshwar 2008). Thus, there is growing interest in the ability to accurately detect HA ranging from high to low molecular weight (LMW). There are several enzyme-linked immunosorbent assay (ELISA)-like methods for the quantification of HA. The most sensitive assays, capable of detecting HA down to 0.2 ng/mL or less, are fluorescence-based assays (Rossler 1998; Martins et al. 2003). Proteoglycans found in...
cartilage, mainly aggrecan and link protein, contain an HA-binding domain that has been widely utilized to make competitive and noncompetitive ELISA-like assays (Tengblad 1979; Chichibu et al. 1989; Kongtawelert and Ghosh 1989; Fosang et al. 1990; Kongtawelert and Ghosh 1990; Agren et al. 1994; Maeda et al. 1999). Hyaluronectin, which is an HA-binding glycoprotein found in the brain, has also been used to make ELISA-like assays for HA (Delpech et al. 1995). The purpose of this study is to determine which commercially available HA ELISA-like assay is able to measure HA ranging from 6.4 kDa to 2 MDa. Additionally, the precision and accuracy of each HA ELISA-like assay will be determined and compared.

Results

Confirmation of the molecular weight of commercially available HA using agarose gel HA sizing

The molecular weight of Lifecore HA (Lifecore Biomedical, Chaska, MN) was verified using an HA sizing gel as described in the “Materials and Methods” section. The expected molecular weights of the Lifecore HA were 6.4, 66 and 132 kDa and 2 MDa. Though the HA sizing gel showed that each size of HA used in the dilution series is actually a smear containing a range of sizes, each smear spans a molecular weight range that includes the expected HA molecular weight (Figure 1A). To confirm that the smears on the agarose gel were HA-specific, half of each sample was digested with Streptomyces hyaluronidase prior to loading as a negative control (Figure 1B). Additionally, based on the smear for the 6.4 kDa HA, it can be concluded that an assay that could not detect 6.4 kDa HA would actually be unable to detect HA under ~27 kDa (Figure 1A).

Ability of HA ELISA-like assays to measure different sizes of HA

A dilution series of each size of HA was made and measured using the Corgenix, Echelon and R&D HA ELISA-like assays. It should be noted that the concentration range of the dilution series varied between the Corgenix, Echelon and R&D assays in order to fall within the standard range of each type of assay, with R&D having a smaller standard range (0.37–270 ng/mL) compared with Corgenix and Echelon (0–800 and 50–1600 ng/mL, respectively). For the Corgenix, Echelon and R&D assays, the sample dilution series ranged from 25–400, 50–800 and 5.63–90 ng/mL, respectively.

Only the Echelon assay was able to detect all sizes of HA tested. The absorbance values of all sizes of HA tested ranged from 0.86 (50 ng/mL HA) to 0.16 (800 ng/mL HA) with a standard deviation of <0.05 for every concentration of HA in the dilution series (Figure 2B). The Corgenix HA ELISA-like assay equally estimated the concentration of all sizes of HA tested except 6.4 kDa HA, with absorbance values of an average of 0.20 (25 ng/mL HA) up to 1 (400 ng/mL HA) and a standard deviation of <0.07 for every concentration of HA tested (Figure 2A). However, the 6.4 kDa HA dilution series had an absorbance value that showed no change regardless of increasing concentration of HA and ranged from 0.14 (25 ng/mL HA) to 0.16 (400 ng/mL HA) with a standard deviation of <0.01 for every concentration of HA tested (Figure 2A). Similar to the Corgenix assay, the R&D assay equally estimated the concentration of all sizes of HA tested except 6.4 kDa HA, with absorbance values of an average of 0.26 (5.63 ng/mL HA) up to 1.32 (90 ng/mL HA) and a standard deviation that ranged from 0.0074 to 0.29 (Figure 2C). The 6.4 kDa HA dilution series had absorbance values that showed no change regardless of increasing HA concentration and ranged

Fig. 1. HA sizing was performed to measure 6.4, 66 and 132 kDa and 2 MDa HA from Lifecore (A). The same samples were digested with S. hyaluronidase as a negative control (B).
from 0.025 (5.63 ng/mL HA) to 0.048 (90 ng/mL HA; Figure 2C).

Accuracy of HA ELISA-like assays
The experimentally determined concentration of the 1:2 dilution series of the commercially available HA of different sizes was compared with the given concentration (Figure 3). The 6.4 kDa dilution series was excluded for the Corgenix and R&D HA ELISA-like assays since neither of these assays were able to detect 6.4 kDa HA. On average, the Echelon assay estimated the HA concentration to be 185% of the concentration determined by the company, whereas Corgenix and R&D estimated the HA concentration to be 68 and 66%, respectively, of the concentration determined by the company (Table I).

HA concentration and intra- and interassay variability of Corgenix, Echelon and R&D HA ELISA-like assays
For experiments which tested the intra- and interassay variability, cell supernatants were collected at a variety of time points from pulmonary artery smooth muscle cells (PASMCs) taken from the explanted lungs of PH patients at the time of lung transplantation. PH PASMCs were chosen because they spontaneously produce high levels of HA including LMW HA (Aytekin et al. 2008). The supernatant pool was measured on three separate occasions for each of the three HA ELISA-like assays tested (Corgenix, Echelon and R&D). The Corgenix assay had the lowest HA measurement ([HA] ± SD ng/mL; 4707.0 ± 281.1), the R&D assay had an intermediate HA measurement ([HA] ± SD ng/mL; 8723.9 ± 2977.5) and the Echelon assay had the highest HA measurement ([HA] ± SD ng/mL; 9400.5 ± 888.9; Figure 4).

Intra-assay variation of the PH PASMC supernatant pool was calculated both for the absorbance values as well as for the HA concentration values in order to account for the effects of dilution on sample variation. In general, the intra-assay variation of the absorbance values of all HA ELISA-like assays tested was lower compared with the intra-assay variation of the HA concentration values (Tables II and III). The intra-assay variability of HA concentration was lowest for the Corgenix assay, intermediate for R&D and highest for Echelon [company, coefficient of variation (CV) ± SD%, n = number of samples/assay; Corgenix, 11.7 ± 3.6, n = 76; R&D, 12.3 ± 4.6, n = 80; Echelon, 18.9 ± 9.2, n = 80; Table II]. The intra-assay variability of the absorbance values was lowest for R&D, intermediate for Corgenix and highest for Echelon [company, CV ± SD%, n = number of samples/assay; R&D, 5.4 ± 1.0, n = 80; Corgenix 7.8 ± 2.4, n = 76; Echelon, 9.6 ± 2.0, n = 80; Table III]. The intra-assay variability of the absorbance values measured by each ELISA-like assay was also calculated with outliers discarded (Tables II and III, respectively). In particular, the intra-assay variability of Echelon decreased significantly when outliers were discarded (CV ± SD%; with outliers: 18.9 ± 9.2; without outliers: 11.8 ± 3.8).

The interassay variability of the HA concentrations for the Corgenix assay was lowest (CV% = 6.0), intermediate for the Echelon assay (CV% = 9.5) and highest for the R&D assay (CV% = 34.1; Table II). The interassay variability of the
absorbance values was lowest for the Corgenix assay (CV% = 9.21), intermediate for the R&D assay (CV% = 15.54) and highest for the Echelon assay (CV% = 53.49; Table III).

Discussion

The goal of this study was to evaluate three commercially available HA ELISA-like assays. The main findings of this work are that the Echelon HA ELISA-like assay is better at detecting very LMW HA (<27 kDa), but the Corgenix and R&D assays have less inter- and intra-assay variability.

A factor in the ability of a HA ELISA-like assay to detect LMW HA is whether two proteoglycans or proteins are used to bind the HA in a sandwich technique as is the case for the R&D ELISA-like assay, in which HA molecules are bound by two recombinant human aggrecan molecules and the Corgenix assay in which two bovine nasal cartilage HA-binding protein (HABP) molecules bind HA or if only one proteoglycan binds the sample HA (as is the case for the competitive Echelon ELISA-like assay). If two proteoglycans are required to bind HA, higher molecular weight HA is needed to accommodate the binding (Li et al. 1989). For instance, when two ELISA-like assays utilizing the same bovine nasal cartilage HA-binding protein (HABP) molecules bind HA or if only one proteoglycan binds the sample HA (as is the case for the competitive Echelon ELISA-like assay). If two proteoglycans are required to bind HA, higher molecular weight HA is needed to accommodate the binding (Li et al. 1989). For instance, when two ELISA-like assays utilizing the same bovine nasal cartilage HABPs were used to measure HA, the assay in which sample HA bound only one HABP was able to detect a minimum of 2 kDa HA whereas the assay in which the sample HA bound two HABPs had a minimum molecular weight of 50 kDa (Chichibu et al. 1989; Maeda et al. 1999).
The accuracy of the three assays was tested by comparing the concentration of Lifecore HA measured by each of the assays to the HA concentration reported by Lifecore Biomedical. These measurements were independent of size, as the 6.4 kDa dilution series was not included in the calculation of accuracy for the R&D and Corgenix assays. The Echelon assay overestimated the HA concentration by 85%, whereas the R&D and Corgenix assays underestimated the HA concentration by 34 and 32%, respectively. Since the exact composition of the HABP utilized for the Echelon assay is proprietary, it is difficult to determine if this difference is due to the binding kinetics of each assay. One possible explanation is that the Echelon standard is less pure than the Lifecore HA, leading to an overestimation of the Lifecore HA concentration. However, this is unlikely since both Echelon (which overestimated) and Corgenix (which underestimated) the Lifecore HA use the same HA standard purchased from Sigma-Aldrich (Minneapolis, MN).

The overestimation of HA concentration by the Echelon kit may be both due to the inherent tendency of the kit to overestimate HA concentration and the ability to detect LMW HA. The inherent overestimation by the Echelon assay is consistent with previous results, as it is stated in the manufacturer’s recommendations: “At times, the values measured using the Echelon Hyaluronic Acid assay have been 2-3 fold higher than those expected from the literature.” The effect of the LMW HA sensitivity of an assay on HA concentration measurements was previously shown when a sample known to contain a high percentage of LMW HA was tested by two different assays. The assay able to detect the LMW HA estimated the HA concentration to be more than 20-fold higher (384 ± 80 vs. 17.9 ± 4.2 ng/mg; Maeda et al. 1999).

Compared with other assays in the literature (Table IV), the intra-assay variability of the HA ELISA-like assays tested in this paper mostly fell within the acceptable limits. The intra-assay variability in previous assays is between 2 and 14% (Table IV). Only one of the assays tested in this paper (Echelon) fell out of that range (CV ± SD%, 18.9 ± 9.2). After 10 outliers were discarded for the Echelon assay (four for trial 1, one for trial 2 and five for trial 3, data not shown), the intra-assay variation fell within the expected range (CV ± SD%, 11.8 ± 3.8; Table II). Some factors that influence the intra-assay variability are amount of samples run, dilution and absorbance shift from left to right across a plate. One report previously determined that a 20% absorbance shift was usually seen from left to right on the assay plates (Delpech et al. 1995). This effect was not seen in the plates used in the experiments presented here (data not shown).

The range for interassay variation in the literature was 3–14% (Table IV). The Corgenix (CV = 6.0%) and Echelon (CV = 9.5%) measurements of HA in the PH PASMC cell supernatant pool fell within this range but the R&D assay (34.1%) was out of this range. The high interassay variability may be explained by the high dilution factor used by the R&D assay (1:200) compared with Corgenix (1:50) and Echelon (1:30). Any small errors in measurement are magnified as the dilution factor increases. When the dilution effect is eliminated, the R&D assay had a more reasonable interassay variation (15.5%). Interestingly, the interassay variation of the absorbance values for the Echelon assay was very high (53.5%), whereas the interassay variation of the HA concentration of the Echelon assay was much lower (9.5%). This can be explained by the way in which the Echelon assay absorbance values are measured. The manufacturer’s instructions are to measure the absorbance at 15, 30 and 45 min until the absorbance of the zero standard is three times greater or more than the absorbance of the 1600 standard after which the stop solution is added. An absolute value for absorbance is not stated. All assays had reached completion by 15 min, but the absolute values of the absorbance varied substantially.

Another possible factor involved in variability is proteins in the cell supernatant that may interfere with the ability of the assays to bind HA. A solution of the proteoglycan A1A1 aggregate produced three times less HA than the same aggregate solution digested with papain prior to the assay to

<table>
<thead>
<tr>
<th>Table II. Intra- and interassay variation in measurements by the Corgenix, Echelon and R&amp;D HA ELISA-like assays of the [HA] in the PH PASMC supernatant pool samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Mean [HA] ± SD (ng/mL)</td>
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<tr>
<td>Intra-assay variation (CV ± SD%)</td>
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<tr>
<td>Interassay variation (CV%)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Table III. Intra- and interassay variation in the absorbance values measured by the Corgenix, Echelon and R&amp;D HA ELISA-like assays with the PH PASMC supernatant pool samples</th>
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<tr>
<td></td>
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<tr>
<td>Mean absorbance value ± SD</td>
</tr>
<tr>
<td>Intra-assay variability (CV ± SD%)</td>
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<tr>
<td>Interassay variability (CV%)</td>
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</table>
degrade proteins (Fosang et al. 1990). It was concluded that the protein digestion released HA bound by proteins and removed proteins from the solution that would compete with the HA ELISA-like assay for HA binding, allowing for a more accurate measurement of HA.

Taken together, the results suggest that each of the three HA ELISA-like assays studied in this paper have different strengths and weaknesses. The Echelon HA ELISA-like assay is better at detecting very LMW HA (<27 kDa). The Corgenix assay had the lowest intra- and interassay variability in HA concentration values, and the R&D assay had the lowest intra-assay variability for the absorbance values. Thus, the selection of the appropriate HA assay should depend on the disease of interest (whether it is expected or known to have LMW HA) and/or the application (need for repeated measures).

**Materials and Methods**

**Preparation of PH PASMC supernatant**

Human PASMCs were isolated from elastic pulmonary arteries (>500 μm diameter) dissected from lungs of PH patients obtained at explanation during lung transplant. After the removal of endothelial cells, PASMCs were dissociated by digestion with collagenase type II/DNAase I solution.

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### Table IV. Comparison of previous HA ELISA-like assays

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Binding protein</th>
<th>Sensitivity</th>
<th>Interassay variation</th>
<th>Intra-assay variation</th>
<th>n</th>
<th>Minimum detectable HA molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncompetitive colorimetric (Li et al. 1989)</td>
<td>Rat chondrosarcoma proteoglycan D1 predigested with chondroitinase ABC (detection with peroxidase-conjugated mouse IgG antibody that binds to keratan sulfate antibody)</td>
<td>15 ng/mL</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>10 kDa</td>
</tr>
<tr>
<td>Noncompetitive colorimetric (Chichibu et al. 1989)</td>
<td>HABP (detection with HRP-avidin D)</td>
<td>2 ng/mL</td>
<td>3.8–10.2%</td>
<td>—</td>
<td>—</td>
<td>50 kDa</td>
</tr>
<tr>
<td>Competitive colorimetric (Delpech et al. 1985)</td>
<td>Hyaluronectin (detection with anti-hyaluronectin antibodies conjugated to alkaline phosphatase)</td>
<td>1 ng/mL</td>
<td>12%</td>
<td>6 ± 2.2%</td>
<td>124 times on eight plates from a patient serum pool</td>
<td>Can detect LMW HA, but molecular weight cut-off not given Decacascaride</td>
</tr>
<tr>
<td>Competitive colorimetric (Goldberg, 1988)</td>
<td>Proteoglycan D1 monomers (detection with peroxidase-conjugated mouse IgG antibody that binds to keratan sulfate antibody)</td>
<td>10 ng/mL</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Competitive colorimetric (Kongtawelert and Ghosh 1990)</td>
<td>Biotinylated HABP</td>
<td>10 pg/mL</td>
<td>19 ± 9%</td>
<td>14 ± 6%</td>
<td>Nine control serum (not listed how many times measured for intra-/interassay variability)</td>
<td>—</td>
</tr>
<tr>
<td>Competitive colorimetric (Delpech et al. 1995)</td>
<td>Alkaline phosphatase-hyaluronectin</td>
<td>&lt;1 μg/mL</td>
<td>8.5% (4°C)</td>
<td>14.4% (4°C)</td>
<td>40 (intra-assay), 2 wells, 15 plates (interassay)</td>
<td>—</td>
</tr>
<tr>
<td>Competitive colorimetric (Fosang et al. 1999)</td>
<td>Biotinylated proteoglycan G1 domain</td>
<td>10 ng/mL</td>
<td>18.5% (37°C)</td>
<td>6.5% (37°C)</td>
<td>Six times in duplicate for intra-assay from a representative urine sample, three different urine samples in duplicate in three assays for interassay</td>
<td>2–10 kDa</td>
</tr>
<tr>
<td>Competitive colorimetric (Maeda et al. 1999)</td>
<td>HABP (detection separate with HRP-avidin D)</td>
<td>&lt;10 ng/mL</td>
<td>8.4%</td>
<td>5.7%</td>
<td>Four healthy control serum samples, one cirrhotic sample measured 10 times each for intra-assay; four healthy and one cirrhotic serum sample measured on five different assays for interassay</td>
<td>—</td>
</tr>
<tr>
<td>Noncompetitive fluorescence (Martins et al. 2003)</td>
<td>HABP (detection with europium-labeled streptavidin)</td>
<td>&lt;0.24 ng/mL</td>
<td>3–14%</td>
<td>2–5%</td>
<td>Four healthy control serum samples, one cirrhotic sample measured 10 times each for intra-assay; four healthy and one cirrhotic serum sample measured on five different assays for interassay</td>
<td>—</td>
</tr>
<tr>
<td>Competitive fluorescence (Rossler 1998)</td>
<td>Cartilage proteoglycan monomer</td>
<td>&lt;0.24 ng/mL</td>
<td>7.02%</td>
<td>3.67%</td>
<td>14 human serum samples, unclear as to how replications were performed</td>
<td>—</td>
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overnight at 37°C (52). Cells were cultured on uncoated plates in the smooth muscle cell growth medium (SmGM-2, Lonza, Allendale, NJ) containing 5% glucose, 10% FBS and 5% antibiotic–antimycotic from GIBCO (Cat no: 15240, Invitrogen, Carlsbad, CA). Cells were passaged at 60–90% confluence by dissociation from plates with 0.05% trypsin, 0.53 mM EDTA; 75 mL of cell supernatant was collected from different PH PASMC cultures, was stored at –20°C until collection was finished, was then combined and mixed for 15 min and aliquoted into 10 mL conical tubes to avoid repeated freeze-thaw.

Preparation of different sizes of HA

The 6.4, 66 and 132 kDa and 2 MDa HA were purchased from Lifecore Biomedical (lot #GSP252-5-7, GSP252-50-2, 002941 and DEV 00453, respectively; Chaska, MN). The size of HA was determined by the company using intrinsic viscosity and the Mark–Houwink equation (Laurent et al. 1960). HA was supplied as a powder. The purity of the HA powder was derived from the high-performance liquid chromatography (HPLC) measurements of HA dissolved in solution compared with a known in-house reference solution. The concentration determined by HPLC was compared with the theoretical HA concentration to calculate purity. The concentration of the following contaminants was tested: endotoxin, bioburden, nucleic acids, protein, osmolality and acetate. Acetate and salts are the most common contaminants. Additionally, due to the hygroscopic nature of HA, water is also important in determining HA concentration, though it is not considered an actual impurity. For all sizes of HA used in this paper, the water content was 5% or less with negligible amounts of other contaminants.

A 10 mL of 5 µg/mL stock solution was made of each size of HA in water. These stocks were used to make dilution series for all the HA size-related experiments in this paper.

HA agarose gel sizing

Ten micrograms of the four different sizes of Lifecore HA solutions mentioned in the section entitled ‘Preparation of different sizes of HA’ were obtained, and half of each size of HA (5 µg) was digested with S. hyaluronidase (100740-1; Seikagaku, Tokyo, Japan) overnight at 37°C. The hyaluronidase was heat killed at 90°C for 10 min. Two 1% agarose (50041; Lonza) gels were prerun at 80 V for 6 h and stored at 4°C until use; 10 M formamide/0.2% bromophenol blue was added to the ±hyaluronidase samples at a ratio of 1:9. Three microliters of 10 M formamide/0.2% bromophenol blue was added to 7 µL of HA molecular weight markers (Select-HATM, Hyalose, Oklahoma City, OK) of mega (6100, 4570, 3050 and 1520 kDa), high (1510, 1090, 966, 572 and 495 kDa) and low (495, 310, 214, 110 and 27 kDa) size. The ±hyaluronidase samples were loaded onto separate gels, each with a set of HA molecular weight markers and subjected to electrophoresis simultaneously at 100 V until the dye front was 80% down the gel. The gels were stained with 0.00625% StainsAll (E9379, Sigma-Aldrich, Saint Louis, MO) overnight on a shaker at room temperature. The gels were de-stained in water for 30 min and then scanned.

HA ELISA-like assays

Assays were purchased from Corgenix (Broomfield, CO) catalog #029-001, lot #HAE-142; Echelon Biosciences (Salt Lake City, UT), catalog #K-1200, lot #ML121809-230; R&D (Minneapolis, MN) catalog #DY3614, lot #1210139. For all assays, the manufacturer’s protocol was followed.

For the different sizes of HA from Lifecore, dilution series were made specifically for each type of assay so that the dilution series would fall within the standard range. For the Corgenix HA ELISA-like assay, each size of HA (5 µg/mL) was further diluted in water to produce the following dilution series: 400, 200, 100, 50 and 25 ng/mL. Following the manufacturer’s instructions, all samples and standards were further diluted at the ratio of 1 part sample or standard to 10 parts Reaction Buffer (provided with the kit) prior to the assay. For the Echelon HA ELISA-like assay, each size of HA (5 µg/mL) was further diluted in Diluent (provided with the kit) to produce the following dilution series: 800, 400, 200, 100 and 50 ng/mL. After this dilution, the samples were ready to load. For the R&D HA ELISA-like assay, each size of HA (5 µg/mL) was further diluted in Reagent Diluent [5% Tween-20 in phosphate-buffered saline (PBS), pH 7.3, 0.2 μM filtered] to produce the following dilution series: 90, 45, 22.5, 11.25 and 5.63 ng/mL. Solutions were mixed at least 5 min on a shaker prior to making the next dilution to ensure equal mixing.

The PH PASMC cell supernatant samples for the Corgenix, Echelon and R&D assays were diluted 1:50, 1:30 and 1:200, respectively. The Corgenix dilution was made by first diluting the samples 1:50 in deionized water and then adding the diluted sample to the Reaction Buffer provided in the kit at the recommended ratio of 1:10 (standards and controls were also diluted 1:10 in Reaction Buffer). The Echelon dilution was made in the provided Diluent. The R&D dilution was made in Reagent Diluent (5% Tween-20 in PBS, pH 7.3, 0.2 μM filtered). All dilutions were mixed at least 5 min to ensure equal distribution of HA and were made in the buffers recommended in the manufacturer’s protocol.

Briefly, the Corgenix HA ELISA-like assay was performed by incubating samples and standards in a 96-well plate that has bovine nasal cartilage HABP immobilized to the bottom of each well for 1 h at room temperature. Bovine nasal cartilage HABP was obtained using a previously established method and is likely to be the link protein and aggrecan G1 domain (Chichibu et al. 1989). The standard was included with the Corgenix assay, though it was originally obtained through Sigma-Aldrich (catalog #H5388, Saint Louis, MO). Sigma supplies this HA standard as a powder and tests it for protein and salt contaminants. The samples and standards were removed by a series of washes and bovine nasal cartilage HABP conjugated to horseradish peroxidase (HRP) was incubated in the plate for 30 min at room temperature. Excess HABP-HRP was removed by a series of washes, and the HRP substrates tetramethylbenzidine and hydrogen peroxide were left on the plate for 30 min at room temperature. The reaction was stopped with 0.36 N sulfuric acid and the absorbance was read at 450 nm, with a 650 nm reading taken as the wavelength correction. The standard curve was graphed using a cubic spline fit. Controls for this assay included a water blank and solutions of previously determined low, medium and high
HA concentration that had the expected absorbance value range listed on each container.

Unlike the Corgenix and R&D HA ELISA-like assays, the Echelon assay utilizes competitive binding. First, samples and standards were mixed with “The Detector”, which is a proprietary, specially modified HABP, and incubated for 1 h at 37°C in the incubation plate. The Echelon assay utilizes the same HA standard as Corgenix. Two-thirds of the samples or standards (obtained from Sigma, catalog #H5388) mixed with “The Detector” were added to the “The Detection Plate” which had HA immobilized on the bottom of each well. Unbound specially modified HABP (“The Detector”) competitively bound to the HA on the bottom of the wells for 30 min at 4°C. The samples, standards and “Detector” were removed through a series of washes, and an alkaline phosphatase able to bind to “Detector” immobilized in the bottom of the wells was incubated on the plate for 30 min at 37°C. The alkaline phosphatase was removed through a series of washes and a p-nitrophenyl phosphate (pNPP) was incubated on the plate in the dark at room temperature. Readings at 405 nm were taken starting at 15 min and once the ratio of the zero HA standard absorbance value was three times greater than the 1600 ng/mL HA standard value, the reaction was stopped with an undisclosed stop solution. All the assays in this paper reached the correct absorbance value ratio after 15 min of incubation. The standard curve was graphed using a four parameter curve and absorbance and sample [HA] were inversely related. A blank containing only assay Diluent was used to correct for background and in all experiments in this paper had an absorbance value of less than the maximum of 0.2.

The R&D HA ELISA-like assay required a room temperature overnight plate-coating step with recombinant human aggrecan. The plate was washed and then blocked with 5% Tween-20, 0.05% sodium azide in PBS for 1 h at room temperature prior to adding. Another series of washes were performed, and samples and standards were added and incubated at room temperature for 2 h. Note that an extra standard was used so that the standards ranged from 0.37 to 270 ng/mL instead of 0.37 to 90 ng/mL. The 270 ng/mL standard is still used so that the standards ranged from 0.37 to 270 ng/mL in the linear range and was chosen to give a wider range of HA concentrations. The HA standard is included with the R&D kit and is supplied as a separate item by R&D as well (catalog #GLR004, Minneapolis, MN). The HA standard is supplied as a powder and is tested for molecular weight (listed as 132 kDa), endotoxins, bioburden, moisture, protein, alcohol and acetate. The samples and standards were removed through a series of washes, and biotinylated recombinant human aggrecan was added to the plate and incubated at room temperature for 2 h. Unbound biotinylated recombinant aggrecan was removed with a set of washes, and the substrate streptavidin-HRP was left on the plate for 20 min at room temperature. The reaction was stopped with 2 N sulfuric acid, and the absorbance was measured at 450 nm with a 540 nm wavelength correction. The standard curve was graphed with using a four-parameter curve.

Caveats to the HA ELISA-like assays

For the Echelon HA ELISA-like assay, the amount of Detector and Diluent provided is small and the use of a multichannel pipette to deliver the Detector to all the wells on the plate is not always possible. For the R&D HA ELISA-like assay, Nunc MaxiSorp™ plates were used. Prior to the experiments in this paper, Nunc PolySorp™ plates were used and it was found that an appropriate absorbance value for the standard curve could not be obtained (data not shown).

Statistical analysis

Statistical analysis was performed in Jmp Version 8 from SAS Institute, Inc. (Cary, NC). The mean (μ) and the standard deviation (σ) were calculated in Jmp Version 8. The CV (expressed as %) values were calculated by the formula:

\[ CV = \frac{\sigma}{\mu} \times 100 \]

Intra-assay variation reported in Tables II and III for the HA measurements in the supernatant pool was determined by calculating the CV value for each assay and then finding the mean and standard deviation of the CV values for all three assays performed per type of ELISA-like assay.

The interassay variation reported in Tables II and III for HA measurements in the supernatant pool were determined by comparing the mean of the three trials performed per type of HA ELISA-like assay (Corgenix, Echelon and R&D). The mean of the absorbance or HA concentration of each trial was calculated, and the CV value was determined from the three means.

Funding

This work was supported by American Heart Association [0826095H to M.A.].

Acknowledgements

We would like to thank Serpil Erzurum and Suzy Comhair for providing the PASMCs, Carol de la Motte for her assistance in finding different sizes of hyaluronan, and Vince Hascall and Mark Lauer for help with the HA sizing technique.

Conflict of interest

None declared.

Abbreviations

CV, coefficient of variation; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; HA, hyaluronan; HABP, hyaluronan-binding protein; HAS, hyaluronan synthase; HRP, horse radish peroxidase; Hyal, hyaluronidase; LMW, low molecular weight; PASMC, pulmonary artery smooth muscle cell; PBS, phosphate-buffered saline; PH, pulmonary hypertension.

References


