PARTIAL PURIFICATION AND DETECTION OF HYALURONAN OLIGO BINDING PROTEIN IN HUMAN BREAST CANCER TISSUE

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INTRODUCTION

HA is a non-sulfated glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine connected by β linkages (Meyer and Palmer, 1934; Fraser et al., 1986; McBride and Bard, 1979; Delmage et al., 1986) and also impedes differentiation, possibly by suppressing cell–cell interactions or ligand access to cell surface receptors. Low molecular weight HA fragments is having distinct and opposing functions from high molecular mass HA. They are mainly 80 and 66 kDa receptors with enhanced expression in malignant breast tumor, which suggested the increased specificity of HA oligosaccharides to its receptors.

MATERIALS AND METHODS

Hyaluronic acid was purchased from Across Organics, New...
Jersey, USA. Sephadex G-50, bovine testicular hyaluronidase type-IS, EDC (1, ethyl 3-(3-dimethyl amino propyl) carbodiimide hydrochloride), MES buffer (2-N-morpholono ethane sulfonic acid) are procured from Sigma chemicals Co, MO, USA. EZ-Link Biotin LC hydrazide was purchased from Thermo Scientific, Rockford, USA and DMSO (Dimethyl sulfoxide) purchased from Himedia. PVDF [Immobilon-p] from Millipore, Massachusetts, USA and HPO-9 (streptavidin peroxidase) from Invitrogen, Carlsbad, CA, USA. Protease inhibitors kit purchased from Invitrogen, USA and ECL kit from Amershan Bioscience, USA.

Methods

Preparation of hyaluronan oligosaccharides

This was carried out according to modified procedure of Banerjee and Toole (1992) by dissolving 100mg of hyaluronic acid in 50mL of 0.05 M CH$_3$COONa containing 0.15 M NaCl (pH 5.0) buffer. 1000 units of bovine testicular hyaluronidase enzyme (type IS, dissolved in same buffer) was added. The reaction mixture was incubated for 24h at 37ºC. The reaction was terminated by boiling for 15min. It was then centrifuged at 10,000 xg for 20 min after which the supernatant was taken and passed through 0.45micron filters. It was lyophilized and then redissolved in minimum amount of triple distilled water and stored at -20ºC until further use.

Characterization of HA-oligosaccharides by FACE analysis

Characterization of HA oligosaccharides is essential to ensure purity and homogeneity prior to experimental study. So fragmentation was checked by fluoro assisted carbohydrate electrophoresis (FACE) essentially according to Sayfried et al. (2005). Both the fractions I and II were derivatized with 2-AA by reductive amination at the reducing end sugar. A 5mL stock solution of 2AA (150mg) and sodium cyanoborohydride (225mg) dissolved in 2% (v/v) acetic acid in methanol was prepared and added to 500µg of above mentioned samples at a 3:1 (v/v) ratio in microcentrifuge tubes. Samples were heated at 80ºC for 45 min to complete the reaction. Excess 2AA was removed by running samples over a Sephadex G-10 desalting column. Purified 2AA-labeled oligosaccharides were diluted with water and 50% (v/v) glycerol to give a final concentration of 16% (v/v) glycerol. Native polyacrylamide of 20% (w/v) containing 0.72% (w/v) methylene bisacrylamide gels (without stacking gel) was made in running buffer composed of 0.1 M Tris-borate, pH 8.4, containing 1mM EDTA. Approximately equimolar amounts of each labeled oligosaccharides were loaded on to 20% native PAGE and ran at 250 V for 1h, after which the gel was viewed under a UV transilluminator.

Preparation of biotinylated hyaluronic acid and hyaluronic acid oligosaccharides probe

500µg of the hyaluronic acid or hyaluronic acid oligosaccharide fragments was dissolved in 500µL of 0.2 M MES buffer (pH 5.5). To this solution, 1mM biotin-LC-hydrazide (dissolved in DMSO) and 10mM EDC were added. The reaction mixture was incubated at 4ºC for 16h. This was dialyzed against PBS-A for 36h at 4ºC. Finally, the dialyzed bHA-oligo was stored in glycerol at –20ºC (Pouyani and Prestwich, 1994).

Tissue homogenization

Fresh samples of malignant tissues (G-III) and benign were collected from Bharath Cancer Hospital, Mysore in cold PBS and stored at –20ºC. Before extraction, the samples were resuspended in lysis buffer and then homogenized [1:4, w/v] using a glass-teflon homogenizer at 4ºC and was centrifuged at 10,000 xg for 45 min at 4ºC. An aliquot of the supernatant was assayed for protein at 280nm in a UV-Shimadzu spectrophotometer.
Western blot analysis to identify tumor receptors

Soluble protein extracts were subjected to 10% SDS-PAGE. Western blotting was carried out according to the modified procedure of Towbin et al. (1979). The gel was transblotted to PVDF membrane and incubating overnight at 4°C with bHA oligosaccharides probe followed by reacted with HPO9 and developed with ECL. The result shown it detected four major binding proteins and the expression was significantly high in cancer breast g-II.

RESULTS

The polymeric HA was digested with hyaluronidase type IS to produce different chain length HA oligosaccharides and were derivatized with anthranilic acid and subjected to 20% native PAGE. The gel viewed under UV-Transilluminator (Fig. 1). The digested mixture showed HA oligo fragments of 8 to 20-mers. The digested HA oligosaccharides and whole HA were conjugated with biotin and dialyzed to remove unbound biotin and were used for further experiments. Western blot analysis was performed by overlay with biotinylated probes (bHA/bHA oligo) to detect the expression of their binding proteins. bHA polymer has recognized multiple binding proteins ranging
HA binding proteins were eluted mostly in 50mM eluted fractions with bHA probe, then reacted with HPO9 and developed with ECL. NaCl eluted fractions of Q-sepharose was transblotted and incubating overnight at 4ºC with bHA probe, then reacted with HPO9 and developed with ECL. HA binding proteins were eluted mostly in 50mM eluted fractions of NaCl eluted fractions. However, moderate reactions with multiple smear bands ranging from 57 to 120 kDa were observed in fibroadenoma (Fig. 2A). While, HA oligosaccharides probe recognized proteins with molecular mass of mainly 80 and 66 kDa in cancer breast g-II (Fig. 2B, lane1), while fibroadenoma expressed mainly 80 kDa band strongly and the expression of 66 kDa protein was significantly reduced. (Fig. 2B, lane 2). Negligible reactions were also found at 57 and 40 kDa in both the tumor samples. To semi-purify the proteins sephadex G-50 column chromatography has performed and has shown 3 peaks (Fig. 3A) and each peak fractions were pooled seperately as F-I, F-II and F-III, and were dialyzed and lyophilized. when the samples subjected to western blot analysis, only the first peak fraction showed the expression of receptors for HA oligosaccharides. The lyophilized first peak fraction was loaded on to a Q-sepharose anion exchange chromatography column and eluted with graded salt concentration of NaCl (Fig. 3B). High amount of protein was recovered in 220mM. All the peak fractions were pooled separetly, dialyzed and concentrated by lyophilization. Western blot analysis with bHA showed differential distribution of HA polymer binding proteins in different salt gradient eluted fractions and major proteins bands were found in 50 mM NaCl eluted fraction (Lane 1, Fig. 4A) and 500mM fraction showed almost no reaction (Lane 5, Fig. 4A). HA oligosaccharides detected protein 80 kDa eluted mainly in 50 and 220 mM fractions, whereas 66 kDa proteins eluted in almost all the fractions (Fig. 4B).

**DISCUSSION**

Hyaluronan oligosaccharides and its receptors have been implicated in tumorgenesis (Sherman et al., 1994; Knudson et al., 1993) but their involvement varies. In this study we attempted to identify and purify HA oligosaccharides receptors expressed in breast tumors. To detect the receptors for HA oligosaccharides, HA polymer was digested and characterized by FACE analysis by conjugated the digested HA with 2-anthranilic acid, a small molecular weight (137.1 Da), highly fluorescent compound. 2-AA not only enhances high performance in gel electrophoreses but also doesn’t disturb the biological activity (Gershkovich and Kholodovych, 1996; Anumala and Dhane, 1998). The FACE analysis showed presence of HA 8 to 20-mers in the digested HA. Western blot analysis (Fig. 2a and 2b) showed HA oligosaccharides recognizing specific receptors when compared to that of hyaluronic acid polymer, which recognized multiple receptors and their expression was relatively high in malignant breast tumor tissue than inflammatory benign tumor (fibroadenoma). In contrast, HA oligosaccharides of 8 to 16-mers also recognized mainly two receptors in human breast and stomach cancers tissues (Srinivas et al., 2012), which are showing the specificity of receptors for HA oligosaccharides. The interaction of Hyaluronan with specific cell surface receptors such as CD44, RHAMM and with intracellular HABP in modulating cellular behavior has been predicted (Banerjee and Toole, 1992; Sherman et al., 1994). The speculation that HA oligosaccharides are involved in tumorogenesis is substantiated with the several findings suggest that HA receptors (HABP) play an important role in tumor metastasis. Large number of receptors (CD44, RHAMM, P-32, TSG-6 etc.) are involved in the progression of cancer, like cell surface and intracellular receptors has been widely implicated in modulating cell behavior.

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**Figure 4A:** Western blot analysis to detect HA binding proteins in Q-sepharose fractions: 50 μg of protein from all NaCl eluted fractions of Q-sepharose was transblotted and incubating overnight at 4ºC with bHA probe, then reacted with HPO9 and developed with ECL. HA binding proteins were eluted mostly in 50mM eluted fractions form 27 to 120 kDa mostly at 120, 80, 66, 57, 50, 40 and 27 kDa and the expression was significantly high in cancer breast g-II. However, moderate reactions with multiple smear bands ranging from 57 to 120 kDa were observed in fibroadenoma (Fig. 2A). While, HA oligosaccharides probe recognized proteins with molecular mass of mainly 80 and 66 kDa in cancer breast g-II (Fig. 2B, lane1), while fibroadenoma expressed mainly 80 kDa band strongly and the expression of 66 kDa protein was significantly reduced. (Fig. 2B, lane 2). Negligible reactions were also found at 57 and 40 kDa in both the tumor samples. To semi-purify the proteins sephadex G-50 column chromatography has performed and has shown 3 peaks (Fig. 3A) and each peak fractions were pooled seperately as F-I, F-II and F-III, and were dialyzed and lyophilized. when the samples subjected to western blot analysis, only the first peak fraction showed the expression of receptors for HA oligosaccharides. The lyophilized first peak fraction was loaded on to a Q-sepharose anion exchange chromatography column and eluted with graded salt concentration of NaCl (Fig. 3B). High amount of protein was recovered in 220mM. All the peak fractions were pooled separetly, dialyzed and concentrated by lyophilization. Western blot analysis with bHA showed differential distribution of HA polymer binding proteins in different salt gradient eluted fractions and major proteins bands were found in 50 mM NaCl eluted fraction (Lane 1, Fig. 4A) and 500mM fraction showed almost no reaction (Lane 5, Fig. 4A). HA oligosaccharides detected protein 80 kDa eluted mainly in 50 and 220 mM fractions, whereas 66 kDa proteins eluted in almost all the fractions (Fig. 4B).

**Figure 4B:** Western blot analysis to detect HA oligosaccharides binding proteins in Q-sepharose fractions: 50 μg of protein from all NaCl eluted fractions of Q-sepharose was transblotted and incubating overnight at 4ºC with bHA oligosaccharides probe, then reacted with HPO9 and developed with ECL. HA oligo binding proteins were distributes in all NaCl eluted fractions.
recognized 80 kDa proteins in lower salt eluted fractions (from 50 to 200mM), while 66 kDa band eluted in almost all fractions indicating specific recognition and distribution of HA oligosaccharide binding proteins. The current study describes differences in the expression of receptor (HABPs) between hyaluronan polymer and hyaluronan oligosaccharides. From the above result it was observed clearly that HA polymer is recognizing multiple HABPs because of its multiple binding sites which exist on the long chain of repeating disaccharide units as compared to HA oligosaccharides. The nature of these HA oligosaccharides receptors are not known. In future the purified HA oligosaccharides receptors will be studied in detail and will be characterized by proteomics analysis and cross reactivity experiments to reveal the nature of these receptors in tumor progression.

REFERENCES


