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Identification of Cell Surface Receptors of HA Oligo-Fragments in Colon Cancer Tissue

[1]S. Manjunath, [2]Faiyaz Ahmed, [3]Geetha Avadni and [1]M. Karuna Kumar

[1]Department of Studies in Biochemistry,
Manasagangotri, University of Mysore, Mysore 570 006 India
[2]Department of Studies in Food Science and Nutrition, Manasagangotri,
University of Mysore, Mysore 570 006 India
[3]Mysore Medical College and Research Institute, Mysore, India

Abstract: HA oligosaccharides are the fragments obtained by the enzymatic degradation of multimolecular hyaluronan. These are angiogenic, inflammatory and immunostimulatory in nature. These interact with specific receptors known as HABPs and thus regulate many cellular functions. How such regulation is accomplished and whether or not these pathways are coordinated with the various sizes specific HA fragments is unknown. In the following study, the different cell surface and cellular receptors for HA oligosaccharides (HA oligomers) in colon cancer tissue have been identified and an attempt has been made to characterize them. Hyaluronic acid was *in vitro* digested followed by separation of specific length oligomers by gel permeation and characterization by their assay and FACE analysis. These were then biotinylated and used as probes to detect the presence of cell surface and cellular receptors in colon cancer tissue by western blotting. Thus, this study was an initiative directed towards understanding the cell surface receptors for HA-oligomers on cancer cells and their further characterization.

Key words: Hyaluronan (Hyaluronic acid, HA) · GAG-glycosaminoglycan · Extracellular matrix (ECM) · HA-oligomers · Hyaluronic acid binding protein (HABP) · Flurophore assisted carbohydrate electrophoresis (FACE)

INTRODUCTION

Hyaluronan (Hyaluronic acid, HA), is a straight chain, non-sulphated glycosaminoglycan polymer of the extracellular matrix composed of repeating units of the disaccharide β(1-3)-N-acetyl-D-glucosamine and β (1-4)-glucuronic acid. Despite the monotony of its composition without branch points or apparent variations in sugar composition. HA has a great number of diverse functions. Hyaluronan promotes cell motility, regulates cell-cell and cell-matrix adhesion, promotes proliferation and suppresses differentiation. It participates in fundamental processes such as embryological development and morphogenesis [1, 2], wound healing [3, 4], repair and regeneration and inflammation [5-7]. Hyaluronan levels increase in response to severe stress and in tumor progression and invasion [8, 9].

At cellular level, it is progressively degraded by the hyaluronidase (Hyal) enzyme family that generates polymers of decreasing sizes. Despite their exceedingly simple primary structure, hyaluronan fragments have extraordinarily wide-ranging and often opposing biological functions. There are large hyaluronan polymers that are space-filling, anti-angiogenic, immunosuppressive and that impede differentiation, possibly by suppressing cell–cell interactions, or ligand access to cell surface receptors. They can also compete with larger hyaluronan polymers for receptors. Low-molecular-size polymers appear to function as endogenous ‘‘danger signals’’, while even smaller fragments can ameliorate these effects. Tetrasaccharides, for example, are anti-apoptotic and inducers of heat shock proteins. How the small hyaluronan fragments are generated is unknown, nor is it established whether the enzymes of hyaluronan synthesis and degradation are involved in maintaining proper polymer sizes and concentration.

HA fragments can bind to HA-binding proteins or hyaladherins [10, 11]. Some of these HA-binding proteins

Corresponding Author: S. Manjunath, Department of Studies in Biochemistry, University of Mysore, Mysore 570 006, India, E-mail: ajaymanju2000@gmail.com.

interact with HA within the extracellular matrix proper, where they are referred to matrix hyaladherins, while others interact with HA at the plasma membrane of the cells, as cell-surface matrix receptors. Such binding has an array of functions, from intracellular effects, such as regulators of the cell cycle [12] or as splicing factors [13]. Extracellular effects are provided by binding to cell surface receptors such as RHAMM and CD44, or to extracellular proteoglycans such as aggrecan and versican. Variations occur in the minimum size of HA oligosaccharides that bind to HA-binding proteins. The HA chain takes on various secondary and tertiary structures that are in part dependent on polymer size. Specific lengths of HA fragments also stabilize or organize arrays of hyaladherins by supporting or inhibiting various combinations of such proteins.

The metabolic pathways for HA synthesis and degradation are highly ordered, composed of carefully controlled reactions that rely on regulation of individual enzyme activities. How such regulation is accomplished and whether or not these pathways are coordinated with the maintenance of size-specific HA fragments is unknown. Currently, HA polymer fragments have widely differing size-specific biological activities. In the present study an attempt has been made to identify the different cell surface and cellular receptors for HA oligosaccharides in cancer tissue involved in tumor progression and invasion.

MATERIALS AND METHODS

Tissue Homogenization: Fresh samples from malignant tissue were collected from the hospitals in cold PBS and stored at -20°C. Before extraction, the samples were resuspended in lytic buffer and then homogenized [1:4 w/v] using a glass-teflon homogenizer at 4°C. The lysate was centrifuged at 8,000 ×g for (45 min) and an aliquot of the supernatant was assayed for protein concentration.

Preparation of Hyaluronan Oligosaccharides: Hyaluronic acid (100 mg) was dissolved in 50 mL of 0.05 M sodium acetate containing 0.15 M sodium chloride. 1000 units of bovine testicular hyaluronidase enzyme type, dissolved in the buffer was added and incubated for 16 h at 37°C. The reaction was terminated by boiling for 15 min. It was then centrifuged at 8,000 ×g (20 min) and the supernatant was taken out and passed through 0.45 micron filters. It was lyophilized and redissolved in small amount of water, concentration was checked using by modified carbazole method and stored at -20°C until further use.

HA Oligosaccharides Assay: The carbazole assay was used to determine the glucouronic acid content of macromolecular HA derived oligosaccharides according to Bitter and Murie using D-glucouronic acid as standard [14]. The reducing N-acetylglucosamine was measured according to the Ressing *et al*. [15] using N-acetyl glucose as standard. This information was used along with its glucuronic acid content to determine the average degree of polymerization of the HA oligosaccharide preparation.

Flurophore Assisted Carbohydrate Electrophoresis (FACE): To confirm fragmentation, FACE analysis was done essentially according to Seyfried *et al*. [16]. Oligosaccharides in the resultant digest mixture were derivatized with 2AA by reductive amination at the reducing end sugar. A 5 mL stock solution of 2AA (150 mg) and sodium cyanoborohydride (225 mg) dissolved in 2% (v/v) acetic acid in methanol was prepared and added to the HA oligosaccharides at a 3:1 (v/v) ratio in microcentifuge tubes. Samples were heated (80°C) for 45 min to complete the reaction. Excess 2AA was removed by running samples over a Sephadex G-10 desalting column. The purified 2AA-labeled oligosaccharides were diluted in water with 50% (v/v) glycerol to give a final concentration of 16% (v/v) glycerol. Native polyacrylamide, 27% (w/v), 0.72% (w/v) methylene bisacrylamide gels (without stacking gel) was made in tris-borate EDTA buffer (pH 8.3). About 30 µg of each labeled oligosaccharide and the digested sample were loaded and run at 300 V, in cold for 45 min. Fragmentation was viewed under UV transilluminator.

Column Chromatography: HA digestion mixture (50 mg) was loaded on a column (85×1.75 cm) packed with G-50 sephadex gel (equilibrated with ammonium acetate buffer) and the flow rate was adjusted to 10mL h^{-1} and the fractions were eluted with the buffer. Fractions were screened for glucuronic and N-acetyl hexosamine. The U/A ratio was then determined and this gave the approximate size of the oligos [17-19]. The peak fractions were pooled as fractions I and II and were lyophilized and redissolved in triple distilled water. F-I was used for further analysis. F-I was again tagged with 2-AA for the FACE analysis.

Preparation of Biotinlyated Hyaluronic Acid Oligosaccharides: Hyaluronic acid oligosaccharides (500 µg) was dissolved in 500 µL of 0.2M MES buffer pH 5.5. To this solution, final concentrations of 1 mM biotin-LC-hydrazide dissolved in DMSO and 10 mM EDC were added and the reaction mixture was incubated at 4°C for 16 h. This was then dialyzed against PBS-A for 36 h at 4°C. Finally, the dialyzed bHA-oligo was stored in glycerol at -20°C. To check the conjugation dot blot was done, where crude protein samples were spotted on the membrane and incubated with biotinylated probe.

Isolation of Tumor Cells by Collagenase Digestion: Cancerous colon was taken into a clean tube under aseptic conditions. To this 2-3 mL of Tyrode solution was added and the tube was shaken vigorously for few minutes. The supernatant was discarded and 1 mL of incubation buffer (pH 7.6) was added along with 1 mg of collagenase and 100 units of BTH. The reaction mixture was incubated at 37°C for 2 h with vigorous shaking for every 15 min. After the incubation period, the tube was centrifuged at 500 ×g. The supernatant was carefully withdrawn and transferred to a clean tube and again centrifuged 3000 ×g for 10 min to get the cells. The supernatant was removed. The pellet was washed with Tyrode solution to remove any debris.

Identification of Cell Surface Receptors: The isolated cell pellet was distributed in equal quantities to 65mm sterile petridishes containing 1ml of the sterilized RPMI-1640 complete media. To this, 15µg of the biotinylated probe F- II was added. The plates were swirled gently for proper mixing and were incubated at 4°C for 2hrs. After the stipulated time, the media of each petriplate was transferred to tubes and were centrifuged to separate the cells. Finally, the cells were homogenized in lytic buffer.

Overlay Experiment to Identify Cellular Receptors: Soluble protein extracts (100 µg) were subjected to 10% SDS-PAGE according to procedure followed by Laemmli *et al.* [20]. The gel was transblotted to PVDF membrane at 200 mA for 45 min at 4°C according to Towbin *et al*. [21]. The membrane was blocked using blocking buffer composed of 5% fat free milk powder, 1% bovine serum albumin, dissolved in PBS. The blot was then incubated with F-1 probe at 4°C overnight, followed by washing with Tris buffer saline containing 0.1% Tween 20 and incubation with horseradish peroxidase for 1h. The complex were detected using an ECL detection kit.

Western Blot Analysis for Cell Surface Receptors: Protein extract from cancer colon cells pre incubated with biotinylated F-I probe,was ran on 10% SDS-PAGE under reducing conditions. PAGE according to followed Laemmli *et el.* [20]. The gel was transblotted to PVDF membrane at 200mA for 45 mins at 4°C according to Towbin *et al*. [21]. The membrane was incubated with 1:20,000 dilution of HPO9 followed by blot development using ECL kit.

RESULTS

FACE analysis of HA oligosaccharides is shown in Figure 1. HA oligosaccharide mixture was prepared and derivatized with 2-AA and used to run a native PAGE. The fragmentation was viewed under the UV light. The results obtained show a typical ladder-like pattern from the digested sample. HA digestion mixture was loaded on G-50 column chromatography, as mentioned in materials and methods section. The pooled fractions-I containing shorter HA fragments were used for further experimentations. These results are depicted in Figure 2.

Figure 3 represents the FACE analysis of F-I HA oligosaccharides. The F-I fraction obtained from the G-50 column was derivatized with 2-AA and was run on a native PAGE for 45 min, showed only 3 clear bands.

Fig. 1: FACE analysis of HA oligosaccharides. Crude HA oligosaccharide digest mixture was prepared as mentioned in methods and materials section, was derivatized with 2-AA and was used to run a native PAGE. The fragmentation was viewed under the UV light

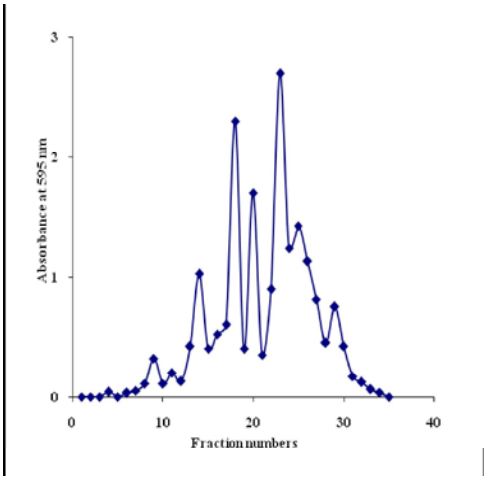

Fig. 2: Gel permeation of digested HA oligosaccharide on Sephadex G-50. 50mg of the HA digestion mixture was loaded on a column (85x1.5cm) packed with G-50 sephadex gel(equilibrated with ammonium acetate buffer)The flow rate was adjusted to 10ml/hr and the fractions were eluted in the buffer. The eluent was pooled to give two fractions- F-I and F-II

Fig. 3: FACE analysis of F-I HA oligosaccharides. The pooled F-I fraction obtained from the G-50 column was derivatized with 2-AA and was run on a native PAGE for 45 mins. The bands were observed under a UV transilluminator

When the FACE analysis profile of the fraction I was compared with that of crude HA digest mixture, it was observed that the purified fraction contains only the smaller HA oligofragments. From the profile, the distinct bands obtained indicate the presence of oligosaccharides in the range 6-10 saccharide units.

Fig. 4: Western blot analysis of cell membrane receptor from colon cancer tissue. Cell extract from cancer colon cells preincubated with biotinylated F-I oligofragments was run on 10% SDS-PAGE under reducing conditions, followed by transblotting, incubation with HPO9 and development by ECL kit

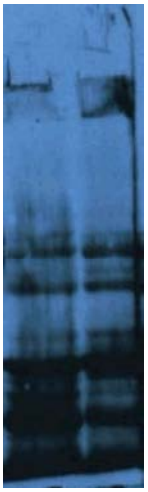

Fig. 5: Western blot analysis of cellular fraction from cancer colon tissue. 100 µg of the soluble protein extracts were subjected to 10% SDS-PAGE under reducing conditions followed by transblotting. The membrane was blocked using blocking buffer and incubated overnight with biotinylated F-I oligofragments. The blot was developed by ECL kit

Further, a single dark band was observed by the Western blot analysis of cell membrane receptor colon cancer tissue (Figure 4). Figure 5 shows the western blot analysis of cellular receptor from colon cancer tissue. A series of bands are observed in the developed blot showing multiple interactions of the HA oligofragments with various HABPs (or receptors) from the cellular fraction of colon cancer tissue.

DISCUSSION

The FACE analysis data obtained the crude digest sample show a typical ladder-like pattern with each band corresponding to a particular length of the oligosaccharide- the topmost faint band indicating undigested HA followed by bottom bands, of relatively higher intensity indicating shorter fragments (hexa, octa and deca units). Due to the long incubation period of the HA polysaccharides with the BTH enzyme (16 h), it has been extensively degraded to give shorter fragments-HA oligosaccharides. On the other hand, the FACE analysis profile of F-I HA oligosaccharides shows only 3 clear bands indicating the presence of smaller HA-oligofragments in the range of 4-12 saccharide units.

Western blot analysis of cell membrane receptor in colon cancer cells, as presented in Figure 5, shows a single dark band. This implies that the fraction I, which consists of smaller HA fragments, have been recognized by a single membrane receptor expressed by the cancer cells of colon. However, the specificity of the receptor is doubtful, because the probe contained a mixture of 4-12oligosaccharides. Therefore, it can be generalized that this receptor recognized HA fragments of length 4-12 oligosaccharide units.

In comparison, a series of bands were observed during the western blot analysis of cellular receptor from colon cancer tissue, showing multiple interaction of the HA oligofragments with various HABPs (or receptors) from the cellular fraction of colon cancer tissue. However, when the same probe was incubated with the whole cells, the binding pattern becomes more specific, showing a single cell surface receptor. Also, the corresponding band for the cell surface receptor in the blot is very faint which might be due to their very low number in comparison to other HABPs.

From these experiments, it maybe concluded that small fragment are generated by enzymatic cleavage in cancer tissue and they are recognized by cell surface receptors in addition to various intracellular HABPs.

HABPs regulate many aspects of cell behavior such as cell-cell adhesion, cell migration and cell differentiation. They also play a role in both the clearance and turnover of hyaluronan in the matrix. Cell surface hyaladherins or HA receptors have been detected on several types of cells and their distribution generally mimics the tissue distribution of HA. The specificity of these receptors, is however a conundrum, as it has been demonstrated that both HA and HA oligofragments can bind to cell surface receptors such as CD44 and RHAMM [22]. Also, ample literature suggest that HA fragment are bioactive in nature and have been implicated to play a role in inflammation and induction of angiogenesis [23]. This gives rise to speculations that there might be receptors for the smaller fragments and binding of the latter may result in signal transduction, through which they mediate their actions. Thus, this study demonstrates the presence of receptors for HA fragments in cancerous tissue, reconfirming the hypothesis that HA oligomers are binding to specific and non-specific receptors [24].

CONCLUSION

The findings of the present investigation paves way for further analysis and experimentation regarding the functions of HA oligosaccharides and their receptors in cancer.

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