The Bioscan 7(4): 727-731, 2012
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

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

SRINIVAS PRASAD KOLLAPALLI[1], SHIB DAS BANERJEE[2] AND KARUNA KUMAR MORTHA*
[1]Department of Studies in Biochemistry, University of Mysore, Manasagangothri, Mysore -570 006 Karnataka, INDIA
[2]Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston, Massachusetts - 02111, USA
e-mail:karunamkk1952@gmail.com

KEY WORDS
Hyaluronan
Breast and Fibroad-enoma

Received on :
10.08.2012

Accepted on :
27.11.2012

***Corresponding author**

ABSTRACT
Hyaluronan is a versatile molecule that can form variety of periodic multimolecular structures through its interaction with specific HA binding proteins. There is increasing evidence showing, HA oligosaccharides expression was associated with malignant progression. HA-oligosaccharides have diverse and complex biological functions both *In vivo* and *In vitro* and are shown to enhance or prevent tumor growth, depending on the size of the fragment through interaction with its receptors. We produced HA 8-20 mers size fragments by enzymatic digestion of HA polymer and characterized by fluorescent assisted carbohydrate electrophoresis. Expression of hyaluronic acid binding proteins for HA polymer and HA oligosaccharides were detected by western blot analysis, which suggested that HA polymer has detected multiple receptors, while HA fragments identified mainly 80 and 66 kDa receptors with enhanced expression in malignant breast tumor, which suggested the increased specificity of HA oligosaccharides to its receptors.

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*., 1989; Lee and Spicer, 2000). HA is a component of extracellular matrix and is present in various tissues and tissue fluids. HA polymers occur in a variety of sizes that have a vast array of properties, some of which appear to be contradictory. The very large HA polymer have an array of regulatory and structural functions like space-occupying, anti-angiogenic, immunosuppressive (Feinberg and Beebe, 1983; 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 angiogenic, inflammatory and immunostimulatory (Stern *et al*., 2006). In general these short oligosaccharides tend to be involved in the body's alarm system, transmitting various modes of ''danger signals'' (Powell and Horton, 2005). HA fragment were shown to recognize Toll like receptor 4 and 2 (TLR4/2) and modulate the inflammatory response in lung (Termeer *et al*., 2002 and Jiang *et al*., 2005). High grade bladder cancers produce 10-15 disaccharide length of HA and it shown mitogenic response/endothelial cell proliferation *in vivo* (Lokeshwar *et al*., 1997), while 6-14 saccharide length HA fragments on human glioblastoma and 10-40 saccharide length HA fragments generated by MIA PaCa shown enhanced CD44 cleavage, proliferation and migration of tumor cells (Sugahara, 2003, 2004 and 2006). HA oligo fragments of 4-6 mers has shown to be involve in upregulation of MMP-9 and MMp-13 expression in Lung cancer cells (Fieber, 2003) and induction of cytokine synthesis in dendritic cells (Termeer *et al*., 2000, 2002). 6-20 monosaccharide size HA fragments stimulate endothelial cell proliferation/angiogenesis (Rooney *et al*., 1993), adhesion and migration by activating the focal adhesion kinase and MAP kinase pathways. Recently HA-oligosaccharide (size 10 disaccharides unit) have been shown to prevent and reduce tumor of malignant gliomas, glioblastoma multiform, in vivo by enhancing apoptosis and down regulating cell survival mechanism, such as, activities of PI3k, RTK, EGFR and c-MET, probably by inhibiting the attachment of multivalent HA polymer to its specific receptors CD44 (Glig *et al*., 2008). The binding of HA oligosaccharides may be different from HA-polymer binding to aggrecan, link protein, versican by virtue of incomplete competition by small oligosaccharides (Hascall and Heinegaard, 1974). Variations occur in the minimum size of HA oligosaccharides binding to HABP's and also, specific length of HA fragments stabilize or organize arrays of hyaladherins by supporting or inhibiting various combinations of such proteins (Stern *et al*., 2006). More information is necessary to understand how various sizes of HA-oligosaccharides and its receptors can influence the vital functions during tumor progression. Due to diverse functions of HA oligosaccharides an attempt has been made to prepare HA 8 to 20-mers and to screen breast tumor tissue to detect HA oligosaccharides binding proteins by western blot analysis and their relative purification by conventional column chromatography.

MATERIALS AND METHODS

Hyaluronic acid was purchased from Across Organics, New

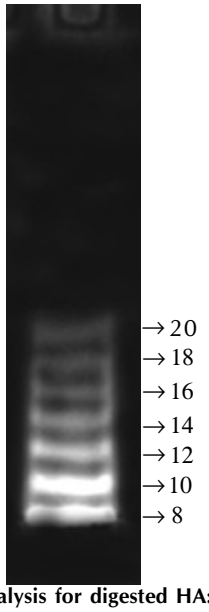

Figure 1: FACE analysis for digested HA: digested hyaluronan was derivatized with 2-AA and run at 200 V on 20% Tris-borate native PAGE. The fragmentation was viewed under the UV light. The digested HA containing HA 8 to 20-mers

Jersey, USA. Sephadex G-50, bovine testicular hyaluronidase type-IS, EDC [1, ethyl 3-(3-dimethyl amino propyl) carbodimide 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_3COONa 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 2AA 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 microcentifuge 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.

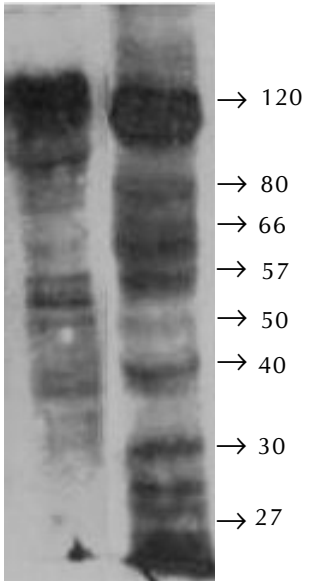

Figure 2A: Western blot analysis to detect HA polymer binding proteins using bHA as a probe: western blot analysis was performed to detect the HABPs from fibroadenoma and ca.breast g- II (lane 1 and 2) by transblotting 50μg crude protein tissue extracts and incubating the blot overnight at 4ºC with bHA polymer, then reacted with HPO9 and developed with ECL. bHA probe detected multiple hyaluronic acid binding proteins and their significant increase in the cancer breast than the fibroadenoma tissue protein extracts

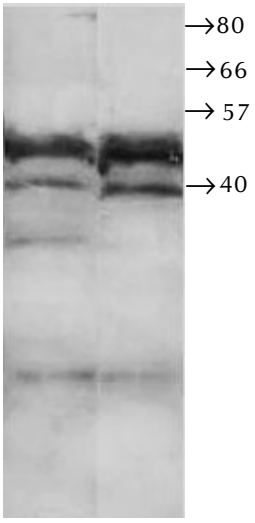

Figure 2B. Western blot analysis to detect HA oligo binding proteins using bHA oligo probe: western blot analysis was performed to detect the HA oligo binding proteins 50μg crude protein tissue extracts of fibroadenoma and cancer breast g- II (Lanes 1 and 2) were transblotted to PVDF membrane and incubating overnight at 4ºC 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

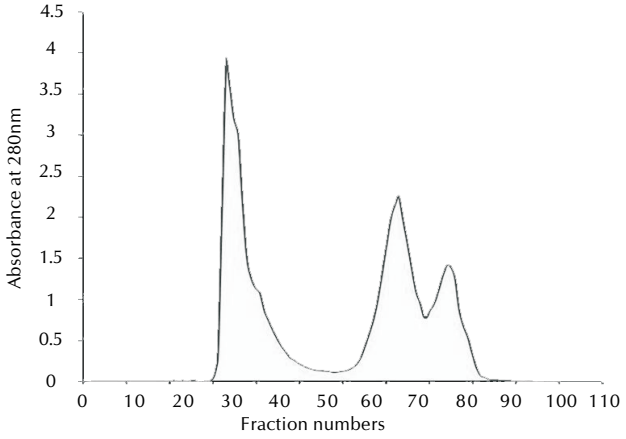

Figure 3A: Elution profile of Sephadex G-50 column chromatography of cancer breast protein extract: 100 mg of crude protein extracted from stomach cancer serum sample was loaded on to a G-50 column (1.5x100cm) and was eluted with elution buffer containing 50mM Tris buffer, pH 8.0. Flow rate was adjusted to 15mL/h. 2mL fractions were collected. Fractions were screened for protein at 280 nm

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 at 200 mA for 45 min at 4ºC. The membrane was blocked using blocking buffer composed of 5% fat free milk powder, 1% bovine serum albumin dissolved in TTBS. The blot was then incubated with biotinylated Hyaluronan or Hyaluronan oligosaccharide probe at 4ºC overnight, followed by washing with Tris buffer saline containing 0.1% Tween - 20 and incubation with HPO9

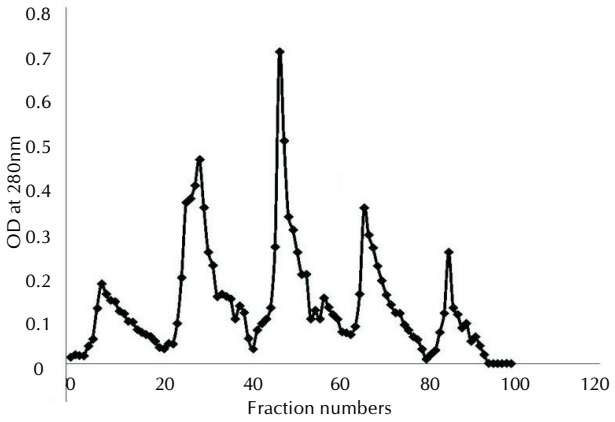

Figure 3B: Elution profile of Q-Sepharose (Ion–exchange column Chromatography) of sephadex G-50 first peak fraction: Approximately 20mg of protein obtained from the first peak of G-50 column was loaded on to a Q-Sepharose column (1.5 x 15cm) and was eluted with elution buffer containing different NaCl concentrations (50, 150, 220, 300 and 500 mM). Flow rate was adjusted to 30 mL/h. 1.0 mL fractions were collected. Fractions were screened for protein at 280 nm

streptavidin peroxidase for 1h. The complex was detected using an ECL detection kit

Gel permeation chromatography) using Sephadex G-50 column

Sephadex G-50 (DNA grade) was swelled in double distilled water for 12h at 4ºC. The gel was packed into a column of bed volume 100mL and equilibrated with 50mM Tris, pH 8.0. The flow rate was adjusted to 15mL/h. Approximately 100mg of crude tissue protein extract was loaded onto the column and the proteins were eluted from the column with 50mM Tris, pH 8.0. Column fractions were read at 280nm absorbance keeping bovine serum albumin as standard. The elution profile was plotted.

Q-Sepharose (Ion exchange chromatography)

Preswelled Q-sepharose (fast flow) was packed in a 10mL column and then equilibrated with 50mM Tris, pH 8.0. The first peak fraction of G-50 column was loaded on to Q-sepharose column. The column is washed with 50mM Tris, pH 8.0 to remove the unbound protein. The proteins were then eluted with 50mM Tris, pH 8.0 containing different salt concentrations (50, 150, 330, 300 and 500 NaCl) sequentially. The eluted fractions were read at 280nm, keeping bovine serum albumin as standard. The elution profile was plotted.

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

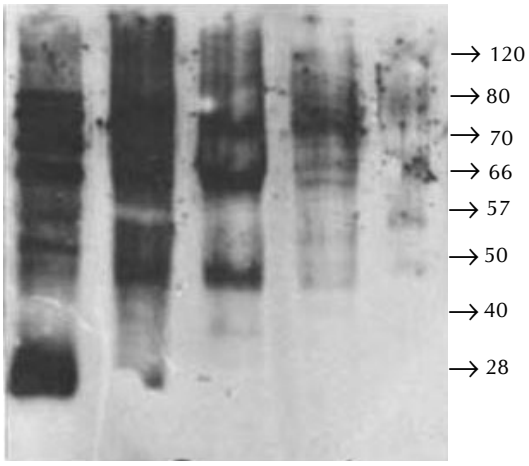

Figure 4A: Western blot analysis to detect HA binding proteins in Q-sepharose fractions: 50 μg of protein from all NaCl eluted fractions of Q-sepharose was transblotted and incubating overnight at 4ºC with bHA probe, then reacted with HPO9 and developed with ECL. HA binding proteins were eluted mostly in 50mM eluted fractions

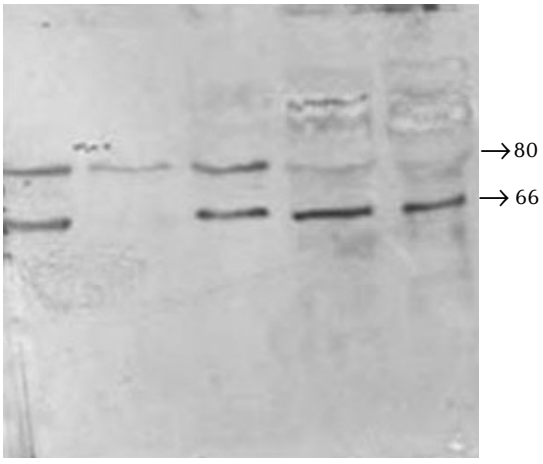

Figure 4B: Western blot analysis to detect HA oligosaccharides binding proteins in Q-sepharose fractions: 50 μg of protein from all NaCl eluted fractions of Q-sepharose was transblotted and incubating overnight at 4ºC with bHA oligosaccharides probe, then reacted with HPO9 and developed with ECL. HA oligo binding proteins were distributes in all NaCl eluted fractions

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). Neglagible 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 seperatley 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 chromatogrpahy 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 saperately, dialyzed and concentrated by lyophilization. Western blot analysis with bHA shown 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 tumorogenesis (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 and play a major role during tumor invasion and metastasis (Toole, 2004; Boregowda *et al*., 2006). When breast Tumor protein extracts were subjected for partial purification on sephadex G-50 chromatography column, it has shown 3 peaks and the first peak fraction showed the binding proteins and the first peak fraction was re-chromatographed on Q-sepharose column to check the distribution of both HA and HA oligosaccharides binding proteins. 50mM eluted fraction showed strong reaction with multiple bands for HA polymer when compared to other salt fractions. However, bHA oligosaccharides

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

Anumula, K. R and Dhume, S. T. 1998. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology*. **8:** 685–694.

Banerjee, S. D. and Toole, B. P. 1992. Hyaluronan-binding protein in endothelial cell morphogenesis. *J. Cell. Biol.* **119:** 643-652.

Boregowda, R. K., Appaiah, N. H., Manjunath, S., Sunil, B. K., Sunila, S., Thimmaiah, K. N., Karuna Kumar, M., Toole, B. P and Banerjee, S. D. 2006. Expression of Hyaluronan in human tumour progression. *J. Carcinog.* **5:** 2.

Delmage, J. M., Powars, D. R., Jaynes, P. K and Allerton, S. E. 1986. The selective suppression of immunogenicity by hyaluronic acid. *Ann. Clin. Lab. Sci.* **16:** 303–310.

Fieber, C., Baumann, P., Vallon, R., Termeer, C., Simon, J. C., Hofmann, M., Angel, P., Herrlich, P. and Sleeman, J. P. 2004. Hyaluronan oligosaccharide induced transcription of metalloproteases. *J. Cell Sci.* **117:** 359-367.

Feinberg, R. N and Beebe, D. C. 1983. Hyaluronate in vasculogenesis. *Science.* **220:** 1177–1179.

Fraser, J. R. E. and Laurent, T. C. 1989. Turnover and metabolism of hyaluronan. In the Biology of Hyaluronan. Ciba Foundation Symp.***143:*** 41-59.

Gershkovich, A. A and Kholodovych, V. V. 1996. Fluorogenic substrates for proteases based on intramolecular fluorescence energy transfer (IFETS). *J. Biochem Biophys Methods.* **33:** 135–162.

Gilg, A. G., Tye, S. L., Tolliver, L. B., Wheeler, W. G., Visconti, R. P., Duncan, J. D., Kostova, F. V., Bolds, L. N., Toole, B. P and Maria, B. L. 2008. Targeting Hyaluronan Interactions in Malignant Gliomas and Their Drug-Resistant Multipotent Progenitors. *Clin Can Res.* **14:** 1804-1913.

Hascall, V. C and Heingard, D. 1974. Aggregation of cartilage proteoglycans. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction. *J. Boil. Chem.* **249:** 4242-4249.

Jiang, D., Liang, J., Fan, J., Yu, S., Luo, Y., Prestwich, G. D., Mascarenhas, M., Garg, H. G., Quinn, D. A., Homer, R. J., Bucala, R., Lee, P. J., Medzhitov, R. and Noble, P. W. 2005. Regulation of lung injury and repair by toll-like receptors and Hyaluronan. *Nature Medicine.* **11:** 1173-1179.

Knudson, C. B. and Knudson, W. 1993. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J.* **7:** 1233-1241.

Lee, J. Y and Spicer, A. P. 2000. Hyaluronan: a multifunctional megaDalton, stealth molecule. *Curr. Opin. Cell. Boil.* **12:** 581-586.

Lokeshwar, V. B., Obek, C., Soloway, M. S. and Block, N. L. 1997. Tumor-associated Hyaluronic Acid: A New Sensitive and Specific Urine Marker for bladder Cancer. *Cancer Res.* **57:** 773-777.

Meyer, K. and palmer, J. W. 1934. The polysaccharide of the viterous humor. *J. Bio Chem.* **107:** 629-634.

McBride, W. H and Bard, J. B. 1979. Hyaluronidase-sensitive halos around adherent cells. Their role in blocking lymphocyte-mediated cytolysis. *J. Exp. Med.* **149:** 507–515.

Pouyani, T. and Prestwich, G. D. 1994. Biotinylated Hyaluronic Acid: A New Tool for Probing Hyaluronate-Receptor Interactions. *Bioconjugate Chem.* **5:** 370 – 372.

Powell, J. D. and Horton, M. R. 2005. Threat matrix: low-molecular-weight hyaluronan (HA) as a danger signal. *Immunol. Res.* **31:** 207–218.

Rooney, P., Wang, M., Kumar, P. and Kumar, S. 1993. Angiogenic oligosaccharides of hyaluronan enhance the production of collagens by endothelial cells. *J. Cell Sci.* **105:** 213–218.

Seyfried, N. T., Blundell, C. D., Anthony, J. D. and Almond, A. 2005. Preparation and application of biologically active fluorescent hyaluronan oligosaccharides. *Glycobiology.* **15:** 303-312.

Sherman, L., Sleeman, J., Herrlich, P. and Ponta, H. 1994. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Current Opin. Cell. Boil.* **6:** 726–733.

Srinivas, P., Kollapalli, S. P., Thomas, A., Mortha, K. K. and Banerjee, S. D. 2012. Bioactive hyaluronan-fragment (hexasaccharide) detects specific hexa-binding proteins in human breast and stomach cancer: possible role in tumorogenesis. *Ind. J. Biochem Biophys.* **49:** 228-235.

Stern, R., Akira, A. A. and Sugahara, K. N. 2006. Hyaluronan fragments: An information rich system. *Eur. J. Cell. Boil.* **85:** 699–715.

Sugahara, K. N., Murai, T., Nishinakamura, H., Kawashima, H., Saya, H. and Miyasaka, M. 2003. Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44 expressing tumor cells. *J. Biol Chem.* **278:** 32259–32265.

Sugahara, K. N., Hirata, T., Murai, T and Miyasaka, M. 2004. Hyaluronan oligosaccharides and tumor progression. *Trends Glycosci Glycotechnol.* **16:** 187–197.

Sugahara, K. N., Hirata, T., Hayasaka, H., Stern, R., Murai, T and Miyasaka, M. 2006. Tumor cells enhance their own CD44 cleavage and motility by generating hyaluronan fragments. *J. Biol Chem.* **281:** 5861–5868.

Termeer, C. C., Hennies, J., Voith, U., Ahrens, T., Weiss, J. M., Prehm, P. and Simon, J. C. 2000. Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J. Immunol.* **165:** 1863–1870

Termeer. C. C., Bendix, F. Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Frewundenberg, M., Galanos, C. and Simon, J. C. 2002. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J. Exp. Med.* **195:** 99-111.

Toole, B. P. 2004. Hyaluronan: from extracellular glue to pericellular cue. *Nat. Rev. Cancer.* **4:** 528–539.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Proc. *Natl. Acad. Sci. USA*. **76:** 4350-4354.