Identification of a Novel Hyaluronic Acid Binding Protein in Human Breast Cancer Using a Monoclonal Antibody

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Abstract: The ubiquitous hyaluronan has diverse biological roles in vertebrates. These include acting as a vital structural component of connective tissues, the formation of loose hydrated matrices that allow cells to divide and migrate (e.g. during development), cell-cell interaction and a role in intracellular signaling. The interaction between hyaluronan and its binding proteins HABP (Hyaladherins) are known to be directly associated with metastasis. Multiple HABP’s have been reported to play a vital role in carcinogenesis. In the present study we have developed a hybridoma and selected a clone H$_2$B$_3$C$_2$ which recognized a very distinct molecular weight protein of 57kDa from breast tumors by western blot. The affinity between the H$_2$B$_3$C$_2$ mAb and HABP was confirmed by reaction with biotinylated HA and competition with HA oligosaccharides. The blocking of the reaction between the H$_2$B$_3$C$_2$ mAb and 57kDa antigen by HA oligosaccharides confirms that this antigen is a hyaluronic acid binding protein and is different from all HABP’s that have been documented earlier.

Key words: Hyaluronan (HA) • Hyaluronic Acid Binding Protein (HABP) • mAb H$_2$B$_3$C$_2$ • breast cancer

INTRODUCTION

Hyaluronan, a high molecular weight polysaccharide found in the extracellular matrix of most animal tissues, has received considerable attention in recent years due to its profound influence on cell behavior [1, 2]. Tissue extracellular matrices become enriched in HA coincident with periods of rapid cell proliferation and migration. Such events have been well documented in developing, regenerating and remodeling tissues and in tissues undergoing malignant tumor cell invasion [2, 3]. HA levels within a matrix have also been shown to promote or inhibit the state of differentiation of several mesenchymal progenitor cell types [2, 4] and to participate directly in cell-cell aggregation events [2, 5]. These matrix-induced effects on cells are in turn supported and directed by a wide variety of HA-binding proteins termed hyaladherins [4]. Some of these HA-binding proteins interact with HA within the extra cellular matrix proper, whereas, others interact with HA at the plasma membrane as cell-surface matrix “receptors” The ability of HA and HA-binding proteins to influence cell physiology and behavior may occur via an alteration of the hydrodynamic and physical properties of the matrix itself or via direct interaction of HA (or its degradation products) with cell-surface protein receptors. The interaction of hyaluronan with matrix hyaluronan-binding proteins and cell-surface hyaluronan receptors regulates many aspects of cell behavior such as cell migration cell-cell adhesion and cell differentiation. Hyaladherins or HA receptors, have been detected on several cell types from a wide variety of tissues [4, 6]. This distribution usually, but not always, mimics tissue distribution of HA [7, 8]. Some reports also suggest that some HA receptors are related or identical to the CD44 family of lymphocyte homing receptors [9]. Other intracellular HA receptors have been reported that do not belong to the CD44 family. Turley and co-workers [10-13] have characterized this HA receptor that mediates HA-
induced cell locomotion. The receptor protein is part of an HA receptor complex (HARC) that occurs both at the cell surface of fibroblasts and released as soluble proteins of molecular weight-72, 68, 58 and 52 kDa. The 58-kDa protein of HARC contains the HA-binding component and has been termed RHAMM, for receptor for HA-mediated motility. Another type hyaladherins that function as intracellular HA receptors are a group of cell-surface antigens recognized by a monoclonal antibody raised by Banerjee and Toole [14] termed IVd4.

These antibodies were raised against a soluble hyaladherin present in embryonic chick brain (unrelated to hyaluronectum and glial-hyaluronan binding protein) and selected by their capacity to block binding of 3H-labeled HA to dot blots of brain extracts. Although these antibodies were raised against soluble antigens, they recognize both extracellular and intracellular HA-binding proteins present on many cell types. Three dominant proteins in the range of molecular weight-50,000-95,000 Dalton have been detected [14, 15]. The relationship between the IVd4-group of hyaladherin receptors and CD44 is not yet clear. Nonetheless, when detected on cells in tissue sections or in culture, these hyaladherin receptors exist most often in an occupied state rather than as unoccupied receptors, suggesting their importance in mediating cell-HA interactions. We have raised a specific monoclonal antibody through extensive clonal selection of IVd4 hybridoma, which recognizes HABP in cancer of human origin. Previous studies showed considerable up-regulation of HA in well differentiated tumors and tumor associated stroma irrespective of their origin, while it is down regulated in poorly differentiated tumors by retaining constant stromal expression [16]. The expression of H1,B1,C2 mAb detected antigen increases as the cancer progresses from well differentiated to poorly differentiated condition irrespective of their cancer origin. Here we report the specific HABP detected in breast grade II tumors by H1,B1,C2 mAb, which is different from all other HABP’s identified so far. Biochemical analysis by immunoprecipitation and immunoaffinity column showed a distinct 57kDa protein characteristic of hyaluronic acid binding protein.

**MATERIALS AND METHODS**

**Chemicals:** Guanidium hydrochloride, Hyaluronidase enzyme solution (Type VI Hyalase Bovine testes) bovine testicular hyaluronidase type VI, Hyaluronan (Na salt human umbilical cord) were procured from Sigma, USA. Streptavidin-horseradish peroxidase conjugated and biotinylated goat antimouse IgG’s (H&L) antibody was purchased from Bangalore, Genei, Karnataka, India. Molecular weight markers were purchased from Fermentas, USA. ECL plus western blotting detection system was purchased from Amersham Biosciences, USA. Blocking buffer: 5% Fat free milk powder, 10% Serum albumin [Bovine], the above reagents were dissolved in PBS. All other chemicals purchased were from Sigma, USA.

**Tissue collection and preparation:** Study consisted of 25 tissue samples from malignant breast tumors accessed from cancer hospitals. The diagnosis was made by the pathologist through routine H&E staining and the tumor was graded using TNM grading system. Breast tumor tissues were brought from hospitals in cold PBS with pH 7.4. They were minced and homogenized in 4X lytic buffer containing 0.2M Tris-HCl (pH 8.0), 80mM EDTA, 0.6M NaCl, 4mM PMSF, 4mM Benzamidine HCl and 2% Triton X-100. The homogenate was centrifuged at 10,000 rpm for 30min at 4°C. The residue was discarded and the supernatant was stored at -20°C until further analysis was carried out. The amount of protein was estimated using Bradford method [17] using Bovine serum albumin as standard.

**Preparation of biotinylated hyaluronic acid:** 50mg of hyaluronic acid was dissolved in 10ml of filtered PBS-A buffer (Ca and Mg free). The dissolved hyaluronic solution was dialysed against 0.1M MES buffer pH 5.5 for 16hrs at 4°C. Later hyaluronic solution was mixed with 50mM biotin-LC-hydrazide dissolved in DMSO to give a final concentration of 1mM, 50mM EDC was added to give a final concentration of 10mM and incubated for 16hrs at 4°C and then dialysed against PBS-A for 36hrs at 4°C. Finally the dialysed bHA was stored in glycerol at-20°C.

**Preparation of hyaluronan oligomers:** This was done according to Banerjee and Toole [2]. 250mg of hyaluronic acid was dissolved in 20ml of 0.05M sodium acetate, 0.15M sodium chloride pH 5.0. To this 3000 units of testicular hyaluronidase predissolved in the above buffer was added and incubated at 37°C for 6hrs. The reaction was terminated by boiling the sample and centrifuged at 12,000rpm for 30min. Then supernatant was collected and it was separated by gel permeation chromatography.

**Production of monoclonal antibody [mAb H1,B1,C2]:** The antibody was originally produced by the fusion of a myeloma variant NS1 with splenic lymphocytes from
SJL/J mice, immunized with semi-purified hyaluronic acid binding protein from 11 days chicken brain [15]. Hybridomas producing IVd4 antibody were selected, whose interaction with antigen was competed out by hyaluronic acid and hyaluronic oligomers [15]. Subsequent hybridomnal clonal selections were performed by heat shock treatment, growing them in bovine serum and finally subcloned in pathogen free human serum received from the hospitals. Further more the hybridoma was selected in HAT and HT media in DMEM. One of the clones H_{i}\_B\_C was selected. The antibody production in human serum of any blood groups did not affect H_{i}\_B\_C antibody in recognizing the human antigen expressed in tissues derived from malignant tumors. The clone H_{i}\_B\_C were grown in DMEM containing 10\% (v/v) human serum. After 14 days the media was collected. The media collected was taken and an equal volume of cold saturated ammonium sulphate solution was added with constant stirring at 4°C overnight and centrifuged at 12000rpm for 30min. The pellet was dissolved in PBS and dialyzed. After dialysis the antibody solution was lyophilized and antibody was dissolved in PBS whenever required.

**Purification of tumor antigen**

**a) HA-sepharose column and purification of HABP:**
100mg of HA was dissolved in sodium acetate containing 0.15M NaCl pH 5.0. To this 2mg of enzyme testicular hyaluronidase (Bovine) was added and incubated for 3hrs at 37°C, then boiled for 10 min. to stop the reaction, centrifuged and supernatant was collected. Digested HA-Sepharose 4B was conjugated according to Green et al. [19]. Then 150mM Nacl fraction from Anion exchange chromatography that showed maximum activity (data not shown) was loaded on to the column and recirculated for couple of times and washed with 3.0M NaCl to remove unbound and eluted the protein with 4M guanidium HCl.

**b) Affinity column chromatography:** 5mg of antibody was dialyzed with coupling buffer for 24hr and allowed to conjugate with CNBr-activated Sepharose 4B for 16hrs at 4°C then it was packed on to a pilot column and unbound was washed with coupling buffer and free sites were blocked with glycine and finally equilibrated with 50mM tris pH 8.0. Extracted breast tumors went through Q-Sepharose and partially purified protein (data not shown) was collected at 150mM NaCl fraction. This fraction was further loaded on to affinity column and eluted with 50mM glycine pH 2.5 and polled separately. The column was washed with 10mM tris pH 8.0 and again eluted with TEA (Tri ethanloamine) pH 11.2, then both samples were dialyzed against 10mM tris pH 7.4 and lyophilized.

**Immunoprecipitation analysis of breast tumor antigen [HABP] and reacted with bHA probe:**
Immunoprecipitation experiment was done according to Green et al. [19]. The 100µg protein from breast cancer samples was immunoprecipitated with mAb H_{i}\_B\_C. Antigen-antibody complex was pulled down by protein-A. Beads were washed extensively and loaded in SDS sample buffer and run on 10% SDS-PAGE. Then proteins were transblotted to PVDF membrane then it was incubated with bHA (1:100 dilution) or incubated with H_{i}\_B\_C antibody (1:100) over night at 4°C. The blot probed with bHA was washed and incubated with streptavidin peroxidase (HP09) for 1 hour at room temperature (1:5000 dilution), for the other blot was incubated with H_{i}\_B\_C antibody and was washed and incubated with goat anti-mouse IgG-biotin conjugated (1:2500 dilution) for 1 hour at room temperature. Later, the membrane was washed and incubated with streptavidin peroxidase (1:5000 dilution) for 1 hour at room temperature, after which they were detected using an ECL Plus Western Blotting Detection System (Amersham Biosciences).

**RESULTS**

**Immunoprecipitation analysis of breast tumor antigen:** To determine the presence of breast tumor antigen in the tissue, 100µg protein from sample was immunoprecipitated with H_{i}\_B\_C mAb and the proteins were separated on a 10% SDS-PAGE and transferred on to a PVDF membrane and reacted with H_{i}\_B\_C mAb (Fig. 1, Lane 1) or reacted with the probe bHA (Fig. 1, Lane 2). The H_{i}\_B\_C mAb
Fig. 1: Immunoprecipitation analysis of H₁₁ antigen and reacted with probe bHA
In this experiment 100µg of protein from cancer breast sample was immunoprecipitated with H₁₁B₂C₂ mAb, transblotted, reacted with H₁₁B₂C₂ mAb (Lane 1) and reacted with probe bHA (Lane 2). The arrowhead indicates the 57kDa HABP.

Fig. 2: Immunoprecipitation analysis of H₁₁ antigen and competition with HA-Oligosaccharides and mAb H₁₁B₂C₂. In this experiment 100µg of protein from cancer breast sample was immunoprecipitated with H₁₁B₂C₂ mAb, transblotted, reacted without HA-Oligosaccharides but with 1:100 dilution H₁₁B₂C₂ mAb (Lane 2) and reacted with 500 µg HA-Oligosaccharides and 1:100 dilution H₁₁B₂C₂ mAb (Lane 1). The arrowhead indicates the 57kDa HABP.

Fig. 3: Western blotting analysis of affinity purified samples
In this experiment purified 57kDa antigen from affinity column chromatography (H₁₁B₂C₂ mAb-CNBr-Sepharose) and HA-Sepharose column were transblotted on to PVDF membrane, reacted with bHA (Lane 1- HA-Sepharose purified and Lane 2- Affinity purified) and reacted with H₁₁B₂C₂ mAb (Lane 3 HA-Sepharose purified and Lane 4- Affinity purified). Lane M shows the molecular weight markers.
recognized protein of molecular weight 57kD (Fig. 1, Lane 1) and probe bHA also recognized protein of molecular weight 57kD, which corresponds to the molecular size of the proteins recognized by the H$_1$B$_2$C$_2$ mAb (Fig. 1, Lane 2). The result shows that tumor antigen recognized by H$_1$B$_2$C$_2$ mAb is present in the cancer breast tissue and the protein present is a hyaluronic acid binding protein is confirmed by reacting with probe bHA.

**Immunoprecipitation analysis of breast tumor antigen and competition with HA-Oligosaccharides and mAb H$_1$B$_2$C$_2$:** To determine that 57kDa antigen present in cancer breast tissue is a hyaluronic acid binding protein, the HA-oligo competition experiment was carried out. 100µg protein from breast cancer tissue sample was immunoprecipitated with H$_1$B$_2$C$_2$ mAb and the proteins were separated on a 10% SDS-PAGE and transferred on to a PVDF membrane and incubated without HA-oligosaccharides and but with H$_1$B$_2$C$_2$: antibody (Fig. 2, Lane 2) incubated with 500 µg HA-oligosaccharides and H$_1$B$_2$C$_2$ antibody (1:100 dilution) (Fig. 2, Lane 1). The result shows the specificity of the H$_1$B$_2$C$_2$ mAb. It was confirmed by using HA-oligosaccharides as explained in the materials and methods section shows the decreased level of the 57kDa antigen when competed with mAb reaction with HA receptors with HA-Oligosaccharides.

**Western blotting analysis of purified breast tumor antigen and reacted with probe bHA:** To determine that purified breast tumor antigen (by affinity chromatography) has a hyaluronic acid binding region, the purified samples were run on 10% SDS-PAGE and transblotted on to a PVDF membrane reacted with H$_1$B$_2$C$_2$: mAb (Fig. 3, Lane 3 and 4) and also reacted with probe bHA (Fig. 3, Lane 1 and 2). The molecular weight markers were also run on the same blot to check the approximate molecular weight of the reacting proteins (Fig. 3, Lane M). The results show that the purified tumor antigen by affinity chromatography (H$_1$B$_2$C$_2$-CNBR-Sepharose and HA-Sepharose) when reacted with bHA and mAb H$_1$B$_2$C$_2$ showed the same 57kD protein concluding that tumor antigen recognized by mAb H$_1$B$_2$C$_2$ has a hyaluronic acid binding region where bHA is reacting and we can confirm that 57kDa antigen is a hyaluronic acid binding protein. The proteins from HA-Sepharose column when reacted with probe bHA showed multiple bands suggesting that HA-Sepharose column is specific to purify hyaluronic acid binding proteins.

**DISCUSSION**

In the present investigation an attempt has been made to identify a hyaluronic acid binding protein, which is recognized by a specific monoclonal antibody H$_1$B$_2$C$_2$. Biochemical analysis with immunoprecipitation and immunoaffinity purified protein showed the expression of breast tumor protein of molecular weight 57kDa recognized by H$_1$B$_2$C$_2$ mAb. The 57kDa antigen is distinct from all other known HABP’s such as CDC 37, IHABP4, HARE, P32, RHAMM etc, even though H$_1$B$_2$C$_2$ clone is originated from Ivd4 hybridoma. This prompted us to investigate the nature of this protein and the extent to which it is a hyaladherin.

To know the nature of 57kDa antigen, H$_1$B$_2$C$_2$: mAb immunoprecipitated protein from cancer breast sample reacted with the probe bHA. This shows that 57kDa antigen is a hyaluronic acid binding protein. Also the tumor antigen is a hyaluronan binding protein was confirmed by the HA-Oligosaccharides competition. 57kDa antigen was partially purified through Q-Sepharose and eluted fractions at 150mM NaCl was further purified by immunoaffinity column eluted with TEA pH 11.2 (H$_1$B$_2$C$_2$-CNBR-Sepharose). Also 150mM Q-Sepharose fraction ran through HA-Sepharose column and eluted with G-HCl. Both eluted fractions then reacted with mAb H$_1$B$_2$C$_2$ showing the same protein at 57kDa. Also bHA interacts with 57kDa protein derived from both HA-Sepharose eluted and immunoaffinity column. This confirms the 57kDa protein is a HABP.

Hyaluronan receptors have been implicated in tumorigenesis [9, 20] but their involvement varies. The interaction of hyaluronan with specific cell surface receptors such as CD44, RHAMM and with intracellular HABP in modulating cellular behavior have been predicted [14, 20]. The speculation that HABP is involved in tumorigenesis is substantiated with the several findings suggest that HA receptors (HABP) play an important role in tumor metastasis. Recently, the identification of hyaluronic acid binding proteins such as CDC37 [21], RHAMM/IHABP [22, 23] and P32 [24] and IHABP4 [25], rising an interesting question.

In the present investigation a new hyaluronic acid binding protein has been identified in grade II breast tumor tissue. The recognition of the 57kDa antigen by biotinylated HA and the ability of the HA oligosaccharides to block the interaction between the H$_1$B$_2$C$_2$: mAb and its antigen indicated that the H$_1$B$_2$C$_2$:reacting 57kDa antigen is a hyaluronan
binding protein. To our knowledge the present investigation is the first biochemical report on the identification of this antigen from grade II breast tumors, which is a hyaluronic acid binding protein. Further studies are underway to check the expression of the protein in different types of tumor tissues and check its regulatory functions in human tumor progression.

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