Introduction

Hyaluronan is known to have various diverse biological functions. HA promotes cell motility, regulates cell-cell interaction, cell-matrix adhesion, cell proliferation and suppresses cell differentiation (Toole, 1991). It not only participates in vertebrates fundamental processes such as embryological development and morphogenesis (Toole, 2001), but is also involved in wound healing (Longaker et al., 1991), wound repair, regeneration and inflammation of tissues (Noble 2002; De la Motte et al., 2003; Majors et al., 2003).

At the cellular level, HA is degraded progressively by the hyaluronidase enzyme family that generates polymers of decreasing sizes. Despite their exceedingly simple primary structure, hyaluronan fragments have extraordinarily wide-ranging and opposing biological functions. Biologically active low molecular size hyaluronan fragments appear to be function as endogenous ‘danger signals’, while even smaller fragments can ameliorate these effects (Stern et al., 2006; Powell and Horton, 2005). HA hexasaccharide, for example, inhibit endothelial cells proliferation (Banerjee and Toole, 1992), while tetrasaccharide are anti-apoptotic and inducers of heat-shock proteins (Xu et al., 2002).

HA fragments also can bind to HA binding proteins or hyaladherins (Toole, 1990; Banerjee and Toole, 1992; Knudson and Knudson, 1993). Some HA binding proteins interact with HA within the extracellular matrix are referred as matrix HA receptors, whereas, others interact at plasma membrane of the cells are known as cell surface receptors. This binding has an array of functions from intracellular effects, such as regulation of the cell cycle, as splicing factors (Deb and Datta, 1996), endothelial cell migration (Banerjee and Toole, 1992; Grammatikakis et al., 1995) etc. 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 current study deals with the detection of HABPs in freshly excised human normal, benign and malignant tumors of ovary and breast using biotinylated hyaluronan polymer, biotinylated HA oligosaccharides (6 to 20-mers), biotinylated HA hexasaccharide, biotinylated HA tetrasaccharide and anti HCAM mAb (CD44s). The identified specific HABPs were also semi-purified by conventional chromatographic techniques.
Results

The expression of HABPs was detected in freshly excised normal endometrium, ovarian tumor (benign) and ovarian cancer tissues by western blot analysis using bHA polymer as a probe. NP-40 extracted crude tissue proteins were resolved on 10% SDS-PAGE and transblotted to PVDF membrane and incubated overnight with bHA polymer. bHA polymer identified proteins with molecular mass of 120, 80, 70, 66, 57, 40 and 27 kDa as HABPs and their expressions were high in ovarian tumor and ovarian cancer than normal endometrium. However, the expression of HABPs was relatively high in ovarian cancer compared to ovarian tumor. These results were depicted in Fig. 4.1. Image J analysis for 80, 66 and 57 kDa proteins showed more density in ovarian cancer (Fig 4.1.1).

Similar experiments were conducted with freshly excised fibroadenoma and breast cancer crude tissue proteins to detect HABPs expression. The results showed expression of multiple HABPs with molecular mass of 80, 70, 66, 57, 40 and 27 kDa and their expressions were enhanced in breast cancer than fibroadenoma. These results were presented in Fig 4.2. Image J analysis for 80, 66 and 57 kDa proteins showed more density in breast cancer (Fig 4.2.1).

Fig. 4.3 shows the expression of HA oligo binding proteins (HOBPs) in normal endometrium, ovarian tumor and ovarian cancer by western blot analysis using bHA oligosaccharides as a probe. NP-40 extracted tissue proteins were resolved in 10% SDS-PAGE and transblotted onto PVDF membrane and incubated with bHA oligosaccharides probe over night. All the tissue samples showed strong expression of proteins at 80 and 66 kDa. But, ovarian tumor and ovarian cancer also showed minor reactions at 57 and 43 kDa protein bands. However, normal endometrium did not expressed 57 and 43 kDa protein bands (lane 1). The expression was comparatively high in ovarian cancer (Lane 3) than normal endometrium and ovarian tumor. Image J analysis for 80 and 66 kDa proteins showed more density in ovarian cancer (Fig 4.3.1).
Western blot analysis of HABP’s expression in normal endometrium, ovarian tumor (benign) and ovarian cancer tissues using bHA polymer probe

Lane 1: Normal endometrium  Lane 2: Ovarian tumor  Lane 3: Ovarian cancer

Western blot analysis was performed to detect the expression of HABPs from normal endometrium, ovarian tumor and ovarian cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA polymer, then reacted with HPO9 and developed with ECL. bHA probe detected multiple HABPs and their expressions were significantly increased in ovarian cancer than ovarian tumor and normal endometrium.
Western blot analysis was performed to detect the expression of HABPs from fibroadenoma and breast cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA polymer, then reacted with HPO9 and developed with ECL. bHA probe detected multiple hyaluronic acid binding proteins and their expressions were significantly increased in the breast cancer than fibroadenoma.
Western blot analysis of HOBPs expression in normal endometrium, ovarian tumor and ovarian cancer tissues using bHA oligosaccharides probe

Lane 1: Normal endometrium  Lane 2: Ovarian tumor  Lane 3: Ovarian cancer

Western blot analysis was performed to detect the expression of HOBPs from Normal endometrium, ovarian tumor and ovarian cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA oligosaccharides, then reacted with HPO9 and developed with ECL. Significant increase in the expression of HOBPs was identified in cancer ovary than ovarian tumor and normal endometrium.
Similar experiments were conducted with fibroadenoma and breast cancer. Though they showed major expression of 80 and 66 kDa proteins as HOBPs, the expression was high in breast cancer (lane 2). In addition, breast cancer also expressed 57 and 43 kDa proteins, while fibroadenoma did not express 57 kDa protein (lane 1). The results were presented in Fig. 4.4. Image J analysis for 80 and 66 kDa proteins showed more density in breast cancer (Fig 4.4.1).

Fig. 4.5 shows western blot analysis of normal endometrium, ovarian tumor and ovarian cancer crude tissue samples with bHA hexasaccharide probe. The tissue protein extracts were resolved on 10% SDS-PAGE and transblotted to PVDF membrane. On overnight incubation with bHA hexasaccharide a doublet band was identified with a molecular mass of 80 kDa only as HA hexasaccharide binding protein (hexa-BP) and the expression was relatively high in ovarian cancer than normal endometrium and ovarian tumor. Image J analysis showed more density of 80 kDa protein in ovarian cancer (Fig 4.5.1).

Similar results were obtained when fibroadenoma and breast cancer crude tissue samples subjected to western blot analysis with bHA hexasaccharide probe. They also expressed a doublet band with a molecular mass of 80 kDa as a specific protein for HA hexasaccharide. The expression of the 80 kDa protein was slightly enhanced in breast cancer. The results were presented in Fig. 4.6. Image J analysis showed more density of 80 kDa protein in breast cancer (Fig 4.6.1).

Fig. 4.7 shows western blot analysis of normal endometrium, ovarian tumor and ovarian cancer crude tissue samples with bHA tetrasaccharide probe. The tissue protein extracts were resolved on 10% SDS-PAGE and transblotted to PVDF membrane. On overnight incubation with HA tetrasaccharide, a doublet band with molecular mass of 80-85 kDa were as tetrasaccharide binding proteins (tetra-BPs) and the expression was relatively high in ovarian cancer than normal endometrium and ovarian tumor. Image J analysis showed more density of 80 kDa protein in ovarian cancer (Fig 4.7.1).
Western blot analysis was performed to detect the expression of HOBPs (for HA 6 to 20-mers) from fibroadenoma and breast cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA oligosaccharides, then reacted with HPO9 and developed with ECL. Breast cancer tissue showed enhanced expression of HOBPs than fibroadenoma.
Western blot analysis of hexa-BPs expression in normal endometrium, ovarian tumor and ovarian cancer tissues using bHA hexasaccharide probe

Lane 1: Normal endometrium    Lane 2: Ovarian tumor
Lane 3: Ovarian cancer

Western blot analysis was performed to detect the expression of hexa-BPs from Normal endometrium, ovarian tumor and ovarian cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA hexasaccharide, then reacted with HPO9 and developed with ECL. Expression of 80 kDa protein was observed and the expression was more in ovarian cancer.
Western blot analysis was performed to detect the expression of hexa-BPs from fibroadenoma and breast cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA hexasaccharide, then reacted with HPO9 and developed with ECL. Expression of 80 kDa protein was observed and the expression was more in breast cancer.
Western blot analysis was performed to detect the expression of tetra-BPs from Normal endometrium, ovarian tumor and ovarian cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA tetrasaccharide, then reacted with HPO9 and developed with ECL. Expression of 80 kDa protein was observed and the expression was more in cancer ovary.
Similar experiments were conducted with fibroadenoma and breast cancer for the detection of HA tetrasaccharide specific protein. These tissues expressed 80 and 85 kDa protein as tetra-BPs and their expressions were relatively high in breast cancer. The results were presented in Fig. 4.8. Image J analysis showed more density of 80 kDa protein in breast cancer (Fig. 4.8.1).

The results of Western blot analysis for bHA hexasaccharide probe pull down with ovarian tumor and ovarian cancer tissue extracts were depicted in Fig. 4.9. The crude NP-40 extracted tissue proteins were incubated overnight at 4°C using bHA hexasaccharide probe and the proteins were precipitated with saturated (NH₄)₂SO₄ and subjected to western blot analysis. Both ovarian tumor and ovarian cancer tissues showed the expression of a doublet band corresponding to 80 kDa protein and their expression was strong in ovarian cancer tissue sample (lane 2) than ovarian tumor. However, ovarian cancer also expressed a minor band at 66 kDa and the expression of 66 kDa protein was negligible in ovarian tumor. Image J analysis showed more density of 80 kDa protein in ovarian cancer (Fig. 4.9.1).

Similar experiments were carried out with fibroadenoma and breast cancer tissue. These results were summarized in Fig. 4.10. Both fibroadenoma and breast cancer tissues showed the expression of a doublet band corresponding to 80 kDa protein and their expression was strong in breast cancer tissue sample (lane 2) than fibroadenoma. However, breast cancer also expressed a minor band at 66 kDa and the expression of 66 kDa protein was negligible in fibroadenoma. Image J analysis showed more density of 80 kDa in breast cancer (Fig. 4.10.1).

In the earlier experiments, 80-85 kDa proteins were identified in all tissue extracts (normal, benign and malignant) and they may be belonging to CD44s family. To confirm whether these proteins belong to CD44s family, western blot analysis was carried out with anti HCAM mAb (CD44s) to detect expression of CD44s. The protein extracts from normal endometrium, ovarian tumor and ovarian cancer were resolved on 10% SDS-PAGE and transblotted to PVDF membrane. On over night incubation with mAb HCAM at 4°C, a doublet band corresponding to molecular mass of 80 kDa was identified and the expression was significantly higher in ovarian cancer. The results were presented in Fig. 4.11. Image J analysis showed more density of CD44s proteins in ovarian cancer (Fig. 4.11.1).
Western blot analysis was performed to detect the expression of tetra-BPs from fibroadenoma and breast cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with bHA tetrasaccharide, then reacted with HPO9 and developed with ECL. Expression of 80 kDa protein was observed and the expression was more in breast cancer.
**Fig. 4.9**

Pull down analysis of hexa-BPs expression in ovarian tumor and ovarian cancer tissues using bHA hexasaccharide probe

Lane 1: Ovarian tumor   Lane 2: Ovarian cancer

250 µg of extracted tissue proteins were pulled down with bHA hexasaccharide overnight at 4°C and precipitated with ammonium sulfate. The pellets were dissolved in 2% SDS and resolved on 10% SDS-PAGE and transblotted to PVDF membrane and reacted with HPO9. The data showed expression of 80 and 66 kDa proteins and their expressions were more in ovarian cancer.
Fig. 4.10
Pull down analysis of hexa-BP expression in fibroadenoma and breast cancer tissues using bHA hexasaccharide probe

Lane 1: Fibroadenoma  Lane 2: Breast cancer

250 ug of extracted tissue proteins were pulled down with bHA hexasaccharide overnight at 4°C and precipitated with ammonium sulfate. The pellets were dissolved in 2% SDS and resolved on 10% SDS-PAGE and transblotted to PVDF membrane and reacted with HPO9. The data showed expression of 80 and 66 kDa proteins and their expressions were more in breast cancer
Western blot analysis was performed to detect the expression of CD44s from normal endometrium, ovarian tumor and ovarian cancer by transblotting 50 μg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with mAb HCAM (CD44). Next day it was treated with goat anti mouse HRP-conjugate antibody and developed with ECL. The expression of CD44s protein was more in ovarian cancer.
Similar experiment was also conducted with fibroadenoma and breast cancer samples to detect the expression of CD44s antigen by western blot analysis using anti HCAM mAb. Both the samples showed expression of proteins with molecular mass of 90 and 80 kDa as CD44s specific proteins and their expression were significantly high in breast cancer (Fig. 4.12). Image J analysis showed more density of CD44s proteins in breast cancer (Fig. 4.12.1).

Crude tissue extracted proteins from normal endometrium, ovarian tumor, fibroadenoma ovarian cancer, and breast cancer were loaded onto a Sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0 with a flow rate of 15 mL/h. The eluted fractions were read at 280 nm for protein. The elution profiles were shown in Fig. 4.13 (normal endometrium), 4.14 (ovarian tumor), 4.15 (fibroadenoma), 4.16 (ovarian cancer) and 4.17 (breast cancer). The profile showed 3 peaks and most of the proteins were eluted in the first peak fractions at void volume. Proteins from the peak fractions separated on 10% SDS-PAGE under reducing condition and transblotted on PVDF membrane and reacted with bHA hexasaccharide or anti HCAM mAb. They showed the proteins of interest were eluted in the first peak (results not shown).

The first peak fractions from sephadex G-50 of normal endometrium, ovarian tumor, fibroadenoma, ovarian cancer, and breast cancer were pooled and lyophilized. The lyophilized first peak fractions were re-chromatographed on a Q-Sepharose (Ion-exchange) chromatography column. The unbound proteins were eluted with 50 mM Tris at pH 8.0. The bounded proteins were eluted with elution buffer 50 mM Tris at pH 8.0 containing graded NaCl concentrations (150, 300 and 500 mM). The flow rate was adjusted to 15 mL/h and 1 mL fractions were collected. Fractions were screened for protein at 280 nm. The elution profiles were depicted in Fig. 4.18, 4.19, 4.20, 4.21 and 4.22 for normal endometrium, ovarian tumor, fibroadenoma ovarian cancer and breast cancer respectively.

Since the main interest of the present work is on HA hexasaccharide, Western blot analysis was carried out to detect the presence of bHA hexasaccharide specific protein from Ion-exchange fractions of normal endometrium. 50 µg proteins from each fraction were resolved on 10% SDS-PAGE and transblotted to PVDF membrane and incubated with bHA hexasaccharide probe. 300 mM fraction showed strong reaction at 80 kDa, while 150 and 500 mM fraction showed no reaction. The results were presented in Fig. 4.23.
Western blot analysis of CD44s antigen (HCAM) expression in fibroadenoma and breast cancer tissues using anti HCAM mAb (CD44s)

Lane 1: Fibroadenoma  Lane 2: Breast cancer

Western blot analysis was performed to detect the expression of CD44s from fibroadenoma and breast cancer by transblotting 50 µg of crude proteins from tissue extracts and incubating the blot overnight at 4°C with mAb HCAM (CD44s). Next day it was treated with goat anti mouse HRP-conjugate antibody and developed with ECL. The expression of CD44 protein was more in breast cancer.
Fig. 4.13
Gel filtration elution profile of normal endometrium tissue proteins using sephadex G-50

Approximately 100 mg of crude proteins from normal endometrium tissue was loaded onto a sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0. Flow rate was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Fig. 4.14  
Gel filtration elution profile of ovarian tumor tissue proteins using sephadex G-50

Approximately 100 mg of crude proteins from ovarian tumor tissue was loaded onto a sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0. Flow rate was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Fig. 4.15
Gel filtration elution profile of fibroadenoma tissue proteins using Sephadex G-50

Approximately 100 mg of crude proteins from fibroadenoma tissue was loaded onto a sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0. Flow rate was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Fig. 4.16
Gel filtration elution profile of ovarian cancer tissue proteins using Sephadex G-50

Approximately 100 mg of crude proteins from ovarian cancer tissue was loaded onto a sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0. Flow rate was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Approximately 100 mg of crude proteins from breast cancer tissue was loaded onto a sephadex G-50 column (1 X 115 cm) and eluted with elution buffer 50 mM Tris, pH 8.0. Flow rate was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Approximately 20 mg of proteins obtained from the first peak of normal endometrium sephadex G-50 was loaded onto a Q-sepharose column (1.5 X 10 cm) and eluted with elution buffer 50 mM Tris (pH 8.0) containing graded NaCl concentrations (150, 300 and 500 mM). Flow through was adjusted to 15 mL/h. 1 mL fractions were collected and screened for proteins at 280 nm.
Approximately 20 mg of proteins obtained from the ovarian tumor sephadex G-50 first peak was loaded onto a Q-sepharose column (1.5 X 10 cm) and eluted with elution buffer 50 mM Tris containing graded NaCl concentrations (150, 300 and 500 mM). Flow through was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Approximately 20 mg of proteins obtained from the fibroadenoma sephadex G-50 first peak fraction was loaded onto a Q-sepharose column (1.5 X 10 cm) and eluted with elution buffer 50 mM Tris containing graded NaCl concentrations (150, 300 and 500 mM). Flow through was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Fig. 4.21
Q-sepharose elution profile of first peak of sephadex G-50 of ovarian cancer tissue (Fig 4.16)

Approximately 20 mg of proteins obtained from ovarian cancer sephadex G-50 first peak fraction was loaded onto a Q-sepharose column (1.5 X 10 cm) and eluted with elution buffer 50 mM Tris containing different NaCl concentrations (150, 300 and 500 mM). Flow through was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Fig. 4.22
Q-sepharose elution profile of first peak of sephadex G-50 of breast cancer tissue (Fig 4.17)

Approximately 20 mg of proteins obtained from the breast cancer sephadex G-50 first peak fraction was loaded onto a Q-sepharose column (1.5 X 10 cm) and eluted with elution buffer 50mM Tris containing graded NaCl concentrations (150, 300 and 500 mM). Flow through was adjusted to 15 mL/h. 1mL fractions were collected and screened for protein at 280 nm.
Western blot analysis of graded salt eluted Q-sepharose fractions of normal endometrium using bHA hexasaccharide probe

Lane 1: 150 mM  Lane 2: 300 mM  Lane 3: 500 mM

50 µg of proteins from each graded salt eluted peak fractions were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with bHA hexasaccharide. Next day it was reacted with HPO9 and developed with ECL. HA hexasaccharide identified 80 kDa protein was mostly eluted in both 300 mM.
Fig. 4.24
Western blot analysis of graded salt eluted Q-sepharose fractions of ovarian tumor using bHA hexasaccharide probe

Lane 1: 150 mM    Lane 2: 300 mM    Lane 3: 500 mM

50 µg of proteins from each graded salt eluted peak fractions were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with bHA hexasaccharide. Next day it was reacted with HPO9 and developed with ECL. HA hexasaccharide identified 80 kDa protein was mostly eluted in both 300 mM.
Fig. 4.25

Western blot analysis of graded salt eluted Q-sepharose fractions of ovarian tumor using bHA hexasaccharide probe

Lane 1: 150 mM      Lane 2: 300 mM      Lane 3: 500 mM

50 µg of proteins from each graded salt eluted peak fractions were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with bHA hexasaccharide. Next day it was reacted with HPO9 and developed with ECL. HA hexasaccharide identified 80 kDa protein was mostly eluted in both 300 mM.
Similar experiments were also conducted with ovarian tumor and fibroadenoma ion-exchange fractions. The results were depicted in Fig. 4.24 and Fig. 4.25. Even though 300 mM fraction showed the major reaction of 80 kDa protein, 150 mM fraction also showed reaction in both the samples.

Fig. 4.26 and 4.27 shows western blot analysis for ovarian cancer and breast cancer graded salt eluted Q-sepharose fractions. bHA hexasaccharide probe detected the presence of 80 kDa protein in both 150 and 300 mM NaCl eluted fractions. However, 300 mM NaCl eluted fraction showed more reaction of 80 kDa protein.

Fig. 4.28 shows the distribution of CD44s antigen in graded salt eluted Q-sepharose fractions of ovarian cancer. Western blot analysis was performed to detect the presence of CD44s in gradient salt eluted Q-sepharose fractions of ovarian cancer. 50 µg of proteins from each fraction (150, 300 and 500 mM) were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with mAb HCAM. Next day it was treated with goat anti mouse biotin conjugate and then treated with HPO9 and developed with ECL. CD44s specific 80 kDa protein was eluted both in both 150 and 300 mM. Hexasaccharide binding protein may be binding with anti HCAM mAb. Both the fractions 150 and 300 mM were pooled together for further experiments.

Fig. 4.29 shows the distribution of CD44s antigen in graded salt eluted Q-sepharose fractions of breast cancer. Western blot analysis was performed to detect the presence of CD44s antigen in gradient salt eluted Q-sepharose fractions of ovarian cancer. 50 µg of proteins from each fraction (150, 300 and 500 mM) were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with mAb HCAM. Next day it was treated with goat anti mouse biotin conjugate and then treated with HPO9 and developed with ECL. CD44s specific 80 kDa protein was eluted both in both 150 and 300 mM. Hexasaccharide binding protein may be binding with mAb HCAM. Both the fractions 150 and 300 mM were pooled together for further experiments.
Fig. 4.26
Western blot analysis of graded salt eluted Q-sepharose fractions of ovarian cancer using bHA hexasaccharide probe

Lane 1: 150 mM  Lane 2: 300 mM  Lane 3: 500 mM

50 µg of proteins from each graded salt eluted peak fractions were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with bHA hexasaccharide. Next day it was reacted with HPO9 and developed with ECL. HA hexasaccharide identified 80 kDa protein was mostly eluted in both 300 mM.
**Western blot analysis of graded salt eluted Q-sepharose fractions of breast cancer using bHA hexasaccharide probe**

Lane 1: 150 mM  
Lane 2: 300 mM  
Lane 3: 500 mM

50 µg of proteins from each graded salt eluted peak fractions were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with bHA hexasaccharide. Next day it was reacted with HPO9 and developed with ECL. HA hexasaccharide identified 80 kDa protein was mostly eluted in both 300 mM.
Western blot analysis was performed to detect the presence of CD44s in gradient salt eluted Ion-exchange fractions of ovarian cancer. 50 µg of proteins from each fraction were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with anti HCAM mAb. Next day it was washed and reacted with goat anti mouse IgG biotin conjugate for 1 h at room temperature followed by reaction with HPO9 and developed with ECL. Anti HCAM mAb identified 80 kDa protein and it was mostly eluted both in 150 and 300 mM.
Western blot analysis was performed to detect the presence of CD44s in gradient salt eluted Ion-exchange fractions of breast cancer. 50 µg of proteins from each fraction were separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was incubated overnight at 4°C with anti HCAM mAb. Next day it was washed and reacted with goat anti mouse IgG biotin conjugate for 1 h at room temperature followed by reaction with HPO9 and developed with ECL. Anti HCAM mAb identified 80 kDa protein and it was mostly eluted both in 150 and 300 mM.
Discussion

The present study was conducted with the understanding that hyaluronan polymer (HA) and its bioactive fragments are shown to be involved directly in tumorigenesis. The current chapter mainly demonstrated the identification of receptors for different size HA oligosaccharides and their subsequent semi-purification by conventional chromatographic techniques.

Because of longer chain length of HA polymer, the more binding sites, HA polymer detected multiple receptors (HABPs) ranging from 120 to 27 kDa and 80 kDa protein was the major receptor (Fig. 4.1 and Fig.4.2). However, HA 6 to 20-mers also has multiple HABP’s with high expression of 80 kDa protein (Fig. 4.3 and Fig. 4.4). It has been shown that HA 6 to 20-mers enhanced CD44 phosphorylation leading to increased tyrosine phosphorylation of endothelial cells (Slevin et al., 1998). Also, inhibit anchorage independent growth of tumor cells by suppressing PI3/AKT pathway through the receptor CD44 (Ghatak et al., 2002).

However, HA 6 and 4-mers also detected 80 kDa protein only (Fig. 4.5 to 4.8). The present data has shown that the avidity to receptor binding was less for HA 4-mer. This is in consistent with the results of Lesley and colleagues (2003) investigation When the tissue proteins subjected to pull down analysis with HA hexasaccharide, once again HA hexasaccharide identified 80 kDa protein and confirmed its binding specificity to 80 kDa protein (Fig. 4.9 and 4.10).

Because, HA polymer and HA oligosaccharide of different size recognized 80 kDa protein, which could be a CD44s specific protein, western blot with anti HCAM mAb (Human Cell Adhesion Molecule or CD44s) were conducted. As usual, anti HCAM mAb detected proteins between 80 to 90 kDa as CD44s specific proteins (Fig. 4.11 to Fig. 4.12).

HA hexasaccharide has been shown to involve in inhibition of endothelial cells proliferation through an unknown receptor (Banerjee and Toole, 1992). However, HA hexasaccharide is the least size HA fragment showed strong reaction with 80 kDa protein, which might be CD44s specific. To understand the relation between the identified 80 kDa protein with CD44s the tissue proteins were semi - purified using gel filtration and Ion-exchange. The receptor for HA hexasaccharide was eluted in the
void volume, the first peak fractions (Fig. 4.13 to Fig. 4.17). When the gel filtration first peak fractions rechromatographed on Ion-exchange, the receptor for HA hexasaccharide eluted in both 150 and 300 mM NaCl eluted fractions (Fig. 4.18 to Fig. 4.27). Parallel experiments with anti CD44 mAb overlay showed that CD44s protein was also eluted in both 150 and 300 mM fractions. Both the fractions were pooled together for further experimental studies (Fig. 4.28 and Fig. 4.29).

The current study describes differences in the expression of receptors between HA polymer, HA oligosaccharide (HA 6 to 20-mers), HA hexasaccharide and HA tetrasaccharide. From the above results it was clearly observed that HA polymer having multiple binding proteins because of its multiple binding sites exists on the long chain of repeating disaccharides compared to HA oligomers, which is having few in number. While, for hexasaccharide and tetrasaccharide, it is still less and having specific site on the specific receptors. However, 80kDa protein is the principle receptor for HA in spite of its size.