CHAPTER I

INTRODUCTION

1.1 STATEMENT OF THIS PROMBLEMS

Cancer is a genetic disorder that usually arises from the accumulation of several mutations. Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation. Continuous division of these cells results in the formation and growth of tumors. A second important process that leads to cancer is that cancerous cells acquire the ability to leave the primary tumor and invade and form colonies at secondary sites. This process, known as metastasis, is caused by changes in the cell-substrate and cell-cell adhesion properties of tumor cells. These cells find a way to dislodge from the tumor into the circulatory system, and travel within the system until they attach themselves to a secondary site. Once attached, the cells activate various molecules, such as membrane-type matrix metalloproteinase (MT-MMP), which allow the cell to invade the tissue at the secondary site (Seiki *et al.*, 2003). When the cells have embedded themselves, they begin to proliferate again and form new tumors; the process of metastasis and secondary site tumor formations keeps repeating and allows the cancer to spread through the organism. (Stubbs *et al.*, 2002)

There is increasing evidence for the role of the extracellular glycosaminoglycan hyaluronan (HA) and its degradative enzymes hyaluronidases (Hyal) in tumor progression. HA is thought to facilitate tumor metastasis by promoting tumor-cell adhesion and migration. Hyal might enhance tumor invasion by facilitating extracellular matrix degradation and tumor angiogenesis. Elevated levels of HA and Hyal have been found in a range of tumors, including those of the prostate, breast and bladder (Yabushita *et al.*, 2005). In the past, quantitative analysis of HA was carried out by separation of that compound from other polysaccharides by diversified techniques followed by colorimetric analysis, such as the carbazole method for uronic acid determination (Scott, 1960; Bitter and Muir, 1962; Galambos, 1967; Hallen, 1972). The discovery that HA can bind specifically to proteins found in cartilage led to the development of a new principle for its specific analysis (Hardingham and Muir, 1972; Hascall and Heinegard, 1974; Hardingham and Adams, 1976; Tengblad, 1980). Subsequently, Tengblad described a radiometric assay based on competition of free HA in solution and HA-substituted Sepharose gel

with HA-binding cartilage protein labeled with ^{125}I (Tengblad, 1980). Thereafter, this assay has been modified for determination of HA in biological samples (Laurent and Tengblad, 1980; Engstrom-Laurent *et al.*, 1985; Brandt *et al.*, 1987) and it was able to measure HA in nanogram amounts. After this original description, several modifications have been made, resulting in a competitive enzyme-linked immunosorbent assay (ELISA) (Delpech *et al.*, 1985; Goldberg, 1988; Kongtawelert and Ghosh, 1989), a competitive fluorescence-based assay (Rossler, 1998), a noncompetitive fluorescence-based assay (Martins *et al.*, 2003), and noncompetitive ELISA-like assays (Chichibu *et al.*, 1989; Li *et al.*, 1989) all maintaining the same principle of the affinity of certain extracellular matrix for HA.

The aims of this study were to develop fluorescence based on competitive ELISA assay and applied for hyaluronan (HA) determination in biological fluids. It was evaluated and then compared concentration of HA in human serum from normal subjects and cancer patients. In present study, it employed HABPs (the globular HA-binding region of the aggrecan and link protein) isolated from chicken cartilage, and FITC conjugated. This technique is simple, fast and has shown precision and sensitivity. Therefore, this assay may be sufficiently suitable for HA measurement.

1.2 LITERATURE REVIEW

1.2.1 Hyaluronan

Hyaluronan, hyaluronic acid, or hyaluronate (HA) is an extracellular polysaccharide, first characterized in the vitreous humor. In 1934, Karl Meyer and co-worker, John Palmer, described a procedure for isolating a novel glycosaminoglycan from the vitreous of bovine eyes. They showed that this substance contained an uronic acid and an aminosugar, but no sulfoesters. The name "hyaluronic acid", from hyaloid (vitreous) + uronic acid. Today, this macromolecule is most frequently referred to as "Hyaluronan".
(http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html)

1.2.2 Structure of Hyaluronan

It would take an additional 20 years before Meyer's laboratory finally completed the work that determined the precise chemical structure of the basic disaccharide motif that forms hyaluronan. During these years they showed that the uronic acid and aminosugar in the disaccharide are D-glucuronic acid and D-N-acetylglucosamine, and that they are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds, (Figure1) (http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html).

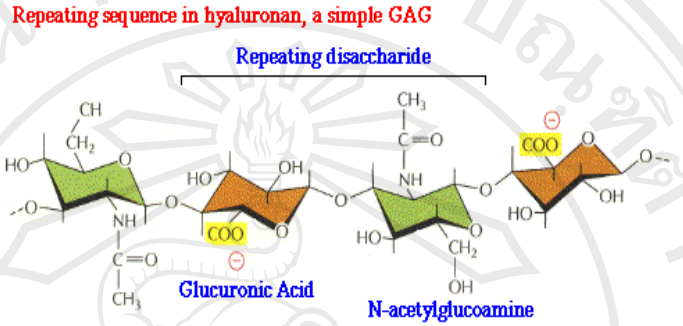

Figure 1. Hyaluronan structure, the repeating disaccharide unit of hyaluronan, contains D-glucuronic acid-beta-1, 3-N-acetylglucosamine-beta-1, 4 glycosidic linkages (http://www.aidp.com/diamond_ series/kolla2-rel.htm).

1.2.3 Hyaluronan and Hyaluronan Binding Protein in Cartilage

Cartilage is a tissue in which the cells (chondrocytes) comprise only a few percent of the volume, and the major part of the tissue is a highly organized and expanded extracellular matrix. The important biomechanical properties of the tissue are the result of the composite structure of the extracellular matrix, which contains: 1) a dense network of fine collagen fibrils (mainly types II, VI, IX and XI), which are responsible for the form and tensile properties of the tissue, and 2) a high concentration of proteoglycan (predominantly aggrecan), which draws water into the tissue by osmosis and exerts a swelling pressure on the collagen network (Figure 2) (Hardingham and Fosang, 1992).

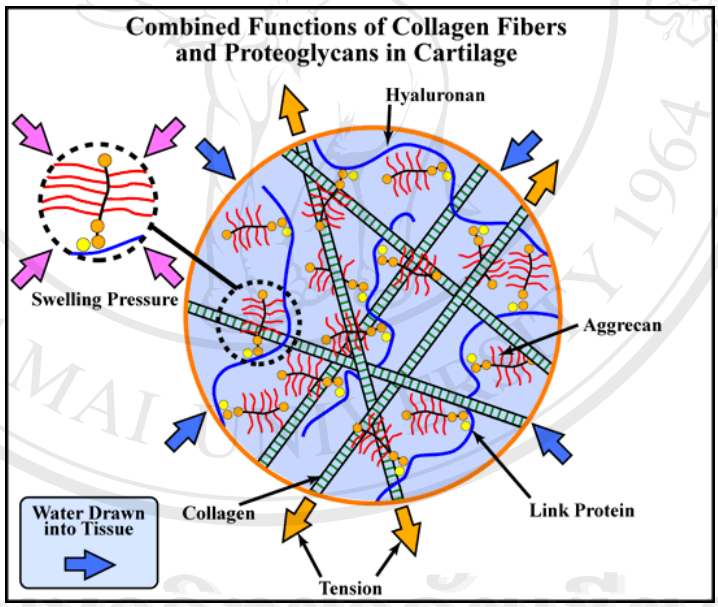

Figure 2. Aggrecan function in cartilage. The aggrecan is immobilized within the matrix by forming supramolecular aggregates with hyaluronan and link protein (Hardingham and Fosang, 1992).

Aggrecan is a proteoglycan with a core protein of high molecular weight (~250,000) encoded by a single gene that is expressed predominantly in cartilaginous tissues. It is highly glycosylated with ~90% carbohydrate, mainly in 2 types of glycosaminoglycan chains, chondroitin sulfate and keratan sulfate (Figure3 and 4) (Hardingham and Fosang, 1992).

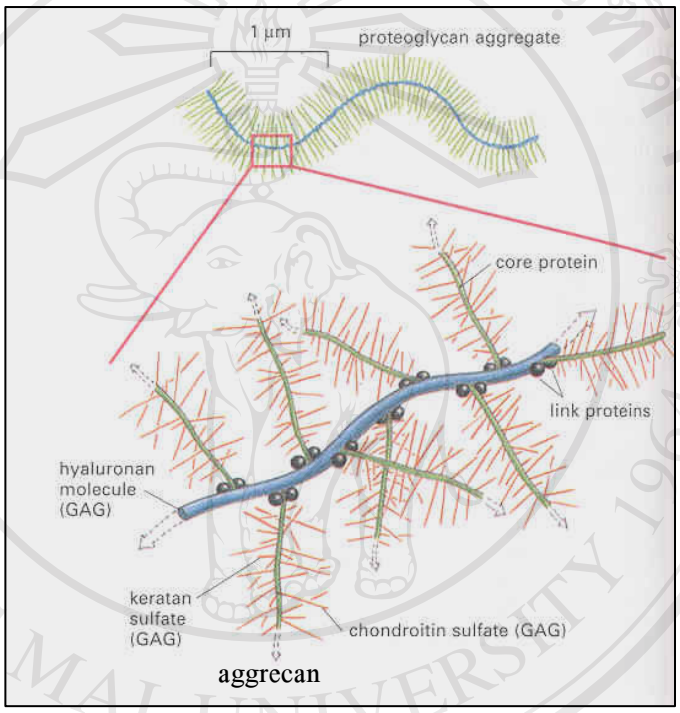

Figure 3. Simple structure of proteoglycan aggregate (http://www.aidp.com/diamond_ series/kolla2-rel.htm).

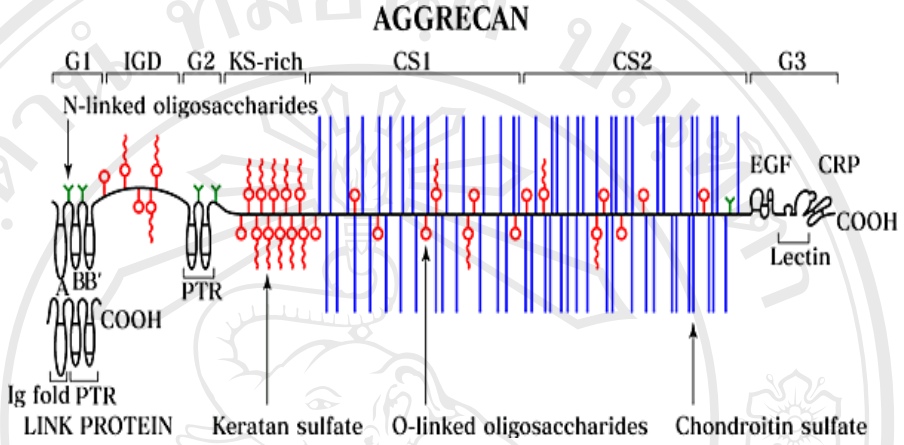

Figure 4. Structure of aggrecan. Aggrecan contains 3 globular domains (G1, G2, and G3) and 2 extended regions, which form the interglobular region between G1 and G2, and the main glycosaminoglycan attachment region. This region is composed of a variable keratan sulfate region and 2 chondroitin sulfate regions (CS-1 and CS-2) distinguished by their sequence patterns. The domain structure of link protein is also shown, which is similar to the aggrecan G1 domain. In aggregates the G1 domain of aggrecan binds to hyaluronan and this binding is stabilized by link protein. PTR (proteoglycan tandem repeat) (Hardingham and Fosang, 1992).

The globular N-terminal G1 domain of aggrecan contains a lectin-like binding site with high affinity for hyaluronan and is responsible for the formation of aggregates. As hyaluronan is a long, unbranched chain with molecular weights of up to several million, each chain can bind a large number of aggrecans to form aggregates up to several hundred million in molecular weight. The binding of each aggrecan to hyaluronan is further stabilized by a small glycoprotein (40-45 kDa) referred to as link protein (Heinegard and Hascall, 1974; Hardingham, 1981). The G1 domain of aggrecan contains 3 protein motifs: an immunoglobulin fold (Ig-fold) and 2 copies of a hyaluronan-binding motif, or link module (also referred to as a proteoglycan tandem repeat).

Link protein has a structure very similar to that of the aggrecan G1 domain, as it contains an Ig-fold and 2 link modules, and this molecular arrangement is also found in the G1 domains of aggrecan, versican, neurocan, and brevican (Figure 5). These proteoglycans form huge, link protein-stabilized complexes with hyaluronan that provide the load-bearing function in articular cartilage, give elasticity to blood vessels, and contribute to the structural integrity of many tissues such as skin and brain (Watanabe *et al.*, 1997; Yamaguchi, 2000). A brain specific link protein (BRAL1) has been characterized recently (Hirakawa *et al.*, 2000), which may be part of a larger link protein gene family (Lee and Spicer, 2000).

Many hyaluronan binding proteins (often termed hyaladherins) contain a common structural domain about of 100 amino acids in length, termed a link module, that is involved in ligand binding (Day, 1999). However, a growing number of hyaladherins lack this domain and are unrelated to each other at the primary sequence level. Some hyaladherins are cell receptors such as CD44 and receptor for HA-mediated motility (RHAMM) and allow cells to interact with the matrix. The protein product of tumor necrosis factor-stimulated gene-6 (TSG-6), which contains a single link module, is secreted in response to inflammatory stimuli (*e.g.,* in the articular joints of arthritis patients)(Day and Prestwich, 2002). Figure 5, 6, 7 and 8 shows the link module superfamily, folds of the link module, other hyaluronan-binding proteins and hyaladherin family.

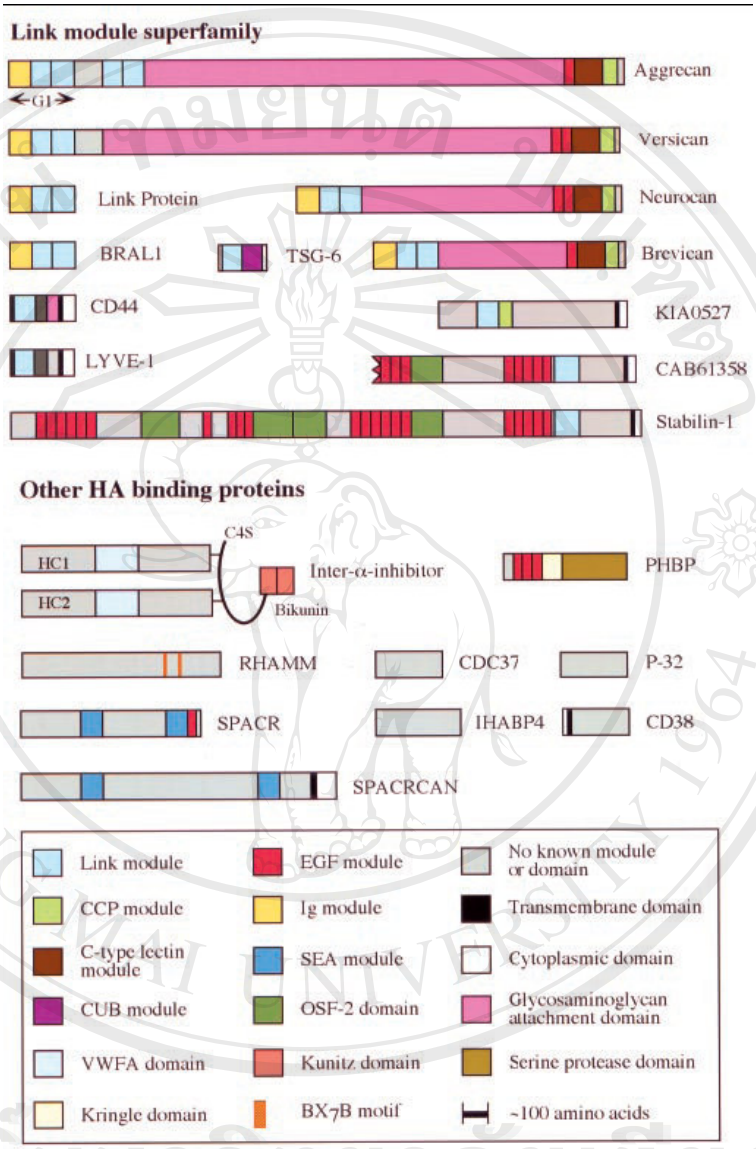

Figure 5. The modular organization of the hyaluronan binding proteins, or hyaladherins (Day and Prestwich, 2002).

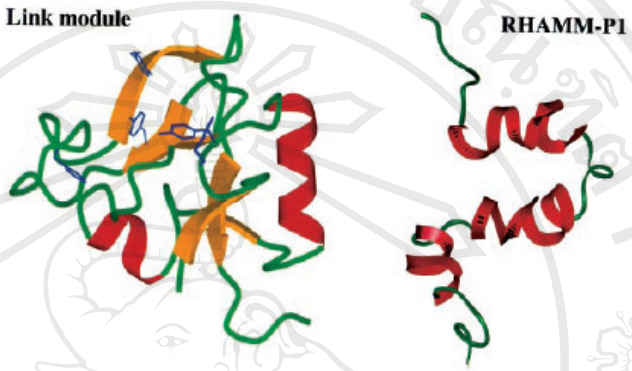

Figure 6. The folds of the link module from TSG-6 (Kohda *et al.*, 1996) (Kohda *et al.*, 1996) and the RHAMM-P1 domain. In the link module, the side chains of residues involved in hyaluronan binding (Mahoney *et al.*, 2001).

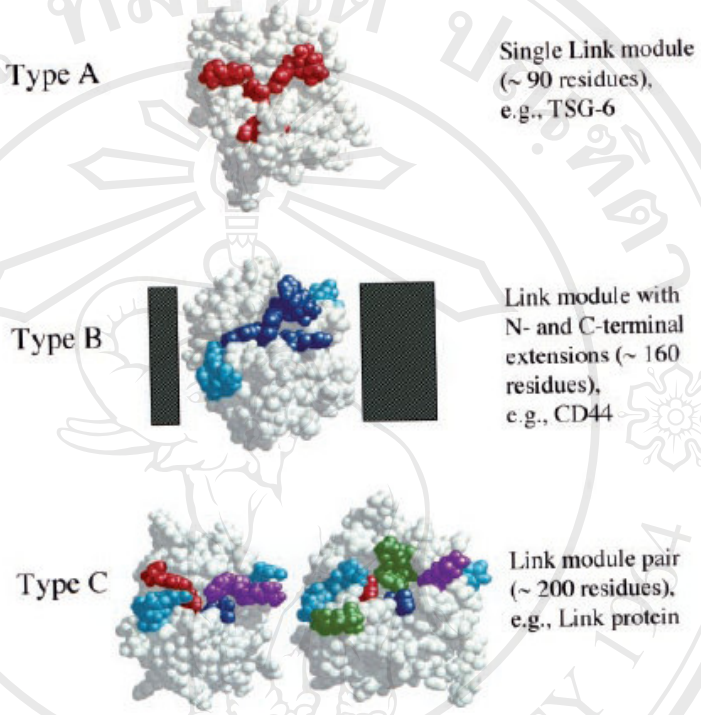

Figure 7. The link module superfamily can be divided into three subgroups on the basis of the size of their hyaluronan-binding domains (Day, 1999).

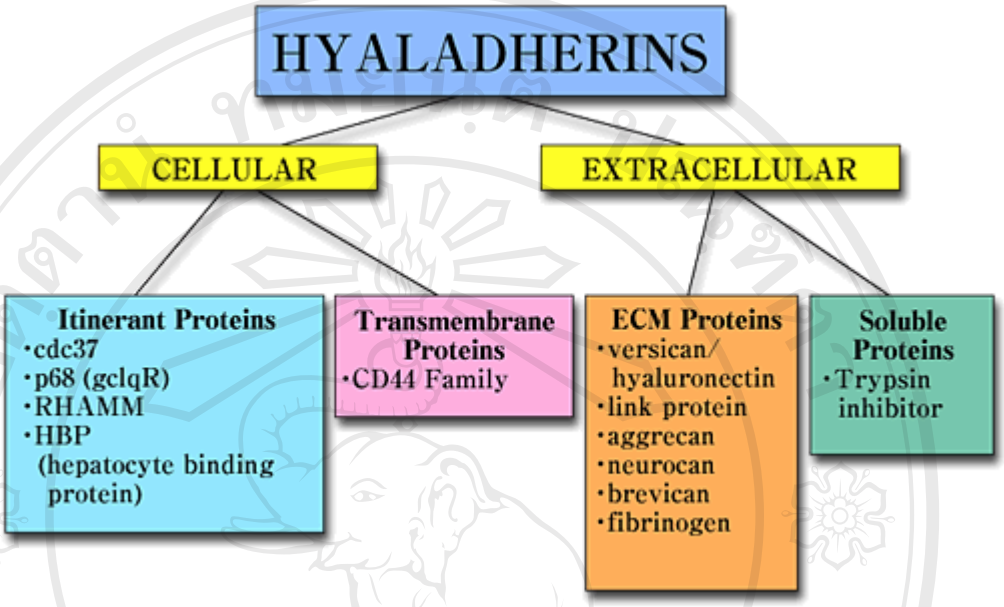

Figure 8. Hyaladherin (or HABP) family of proteins. Hyaladherins are a disparate group of proteins linked by a common ability to bind to hyaluronan. They can be grouped according to their cellular and extracellular location (Entwistle *et al.*, 1996).

1.2.4 Hyaluronan Synthesis

Hyaluronan is synthesized in mammals and bacteria by a family of three hyaluronan synthases (Has1, Has2 and Has3)(Spicer and McDonald, 1998). All three isozymes catalyze the same reaction: that is, successive alternating addition of glucuronic acid and N-acetylglucosamine from the respective UDP esterified sugar precursors, in a repeating disaccharide motif. Hyaluronan synthases are also membrane enzymes. Polymerization is concurrent with secretion of the growing chain to the extracellular space, such that its final size is not constrained by intracellular dimensions. Newly synthesized hyaluronan polymers range in average molecular mass from $1x10^5$ to $1x10^7$ Da (Figure 9).

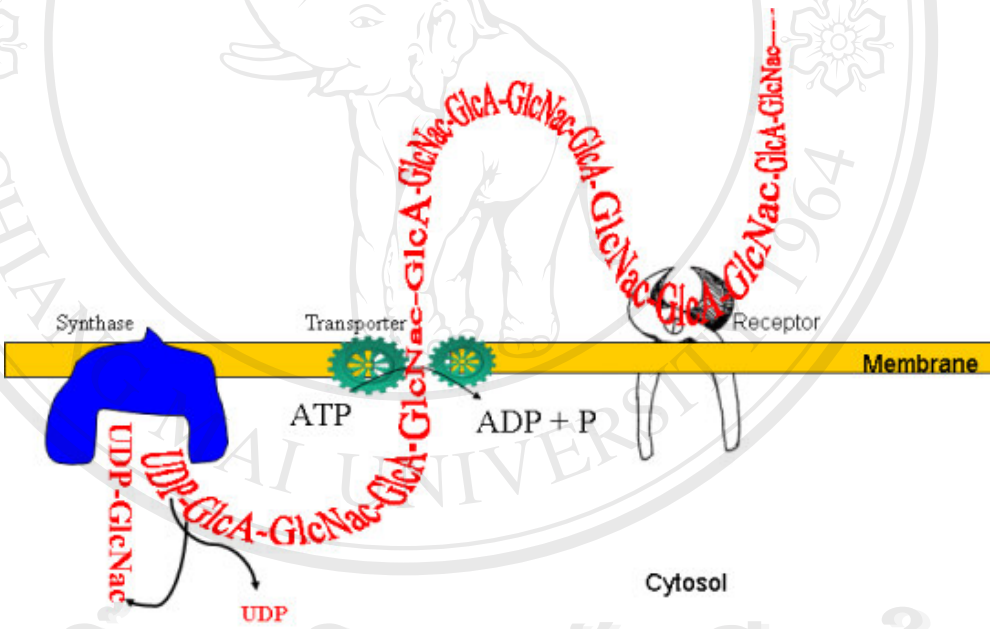

Figure 9. Hyaluronan biosynthesis. Hyaluronan synthesis in mammalian cells differs from other polysaccharides in many aspects. It is elongated at the reducing end by alternate transfer of UDP-hyaluronan to the substrates UDP-GlcNac and UDP-GlcA liberating the UDP-moiety. It is synthesized at plasma membranes and nascent chains are directly extruded into the extracellular matrix. (http://physiolchem.klinikum.uni-muenster.de/mitarbeiter/prehm_research.html)

1.2.5 Turnover and Catabolism of Hyaluronan

Hyaluronan was discovered over 50 years ago but its metabolism and cellular interactions have only recently received detailed attention. Hyaluronan has an extraordinarily high rate of turnover in vertebrate tissues. Tissue HA enters the bloodstream in significant amounts through the lymph and is rapidly absorbed via a receptor into liver endothelial cells, where degradation follows. HA levels in serum are normally 10-100 μg/l, a 70 kg individual has 15 g of hyaluronan, 5 g of which turns over daily, and in the bloodstream, hyaluronan has a half life of two to five minutes, but can be elevated in cirrhosis, rheumatoid arthritis and scleroderma, due either to impaired hepatic uptake or to increased production.

It is well established that hyaluronan is taken up by cells for degradation (Collis *et al.*, 1998; Tammi *et al.*, 2001) through receptors such as CD44 (Culty *et al.*, 1992; Hua *et al.*, 1993; Kaya *et al.*, 1997). The high molecular weight extracellular polymer is tethered to the cell surface by the combined efforts of receptors such as CD44 and the GPI-anchored enzyme hyaluronidase-2 (Hyal-2). The hyluronan-CD44-Hyal-2 complex is enriched in specialized microdomains. These are invaginations of the plasma membranes composed of cholesterol and gangliosides, termed lipid rafts, significant because they also recruit a large number of key signaling molecules. One category of lipid rafts is caveolae, structures rich in the proteins caveolin and flotillin. The hyaluronan polymer is then cleaved to 20 kDa limit products(Lepperdinger *et al.*, 1998), corresponding to about 50 disaccharide units. The Hyal-2-generated hyaluronan fragments are internalized, delivered to endosomes, and ultimately to lysosomes, where Hyal-1 degrades the 20 kDa fragments to small disaccharides. Two lysosomal β-exoglycosidases, β-glucuronidase and β-N-acetyl-glucosaminidase, participate in this degradation. An overall scheme for HA catabolism in diagrammatic form is presented below (Figure 10 and11).

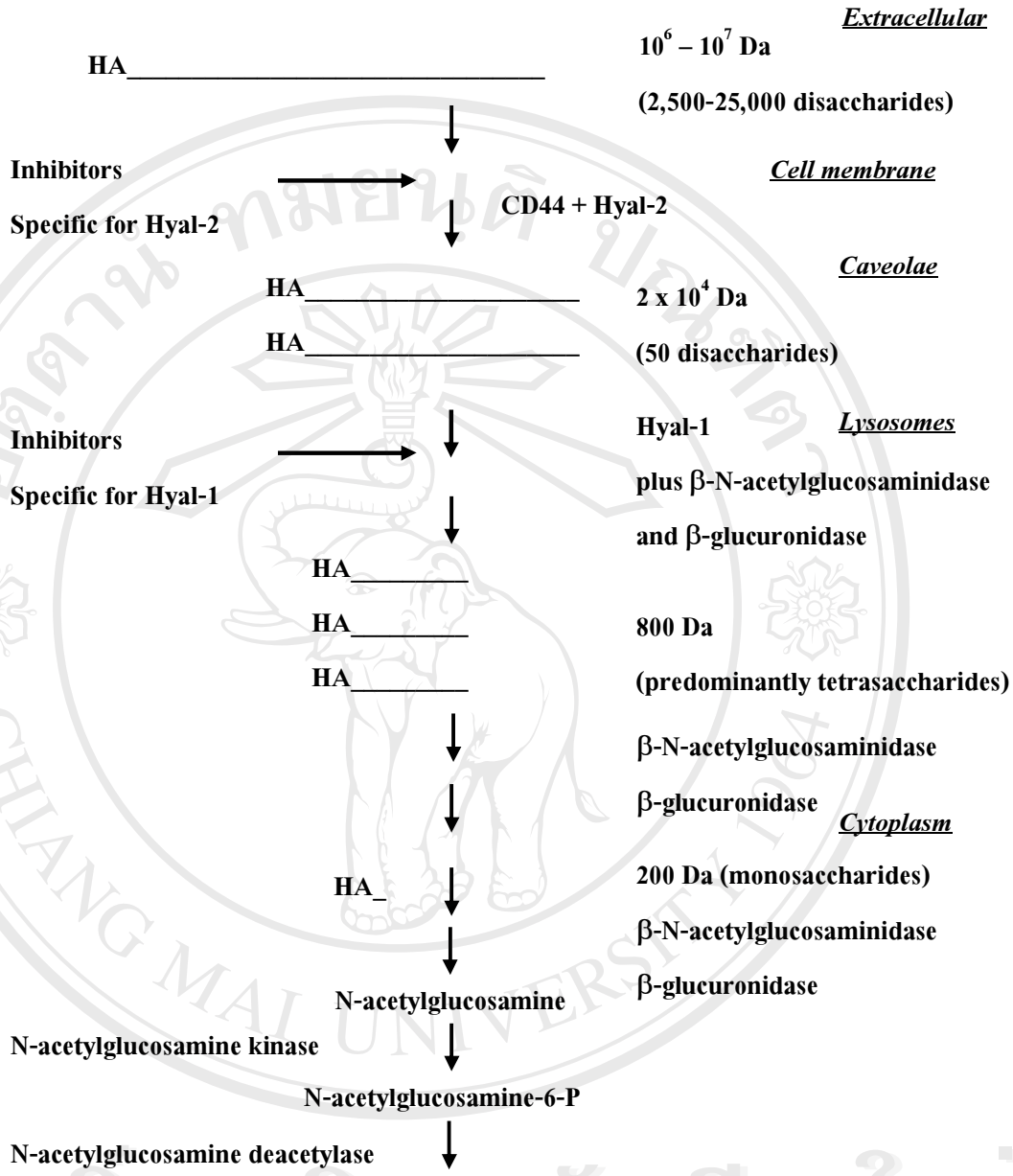

Figure 10. The putative scheme for HA catabolism, beginning with a high molecular weight extracellular polymer, and ending with single sugars that are then available for other metabolic cycles (Stern, 2004).

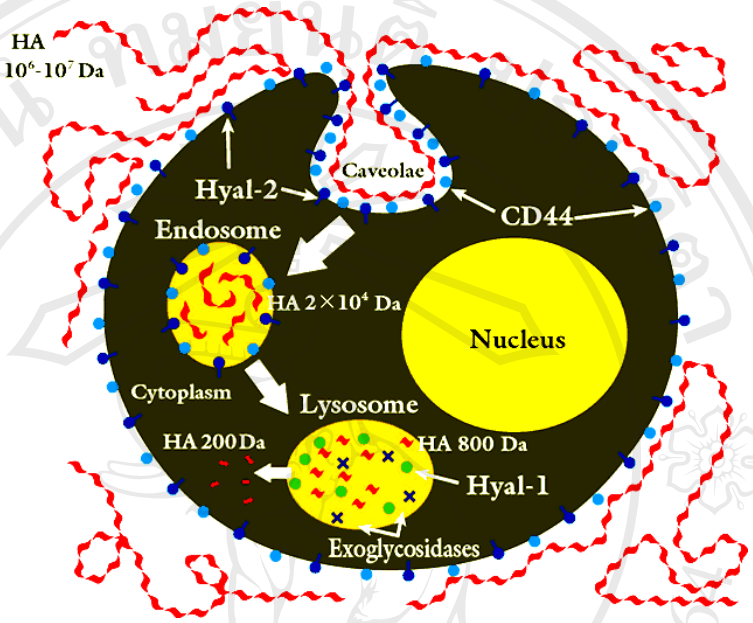

Figure 11. Diagram of a putative metabolic scheme for hyaluronan degradation (Stern, 2004) (http://www.glycoforum.gr.jp/science/hyaluronan/HA15a/HA15aE.html).

1.2.6 Physiological and Biological Function of Hyaluronan

Hyaluronan in tissues, hyaluronan is present in all vertebrates, perhaps arising in animals with notochords. It is also present in the capsule of some strains of Streptococci that quite likely pirated the enzymatic machinery for its synthesis from vertebrate hosts. Hyaluronan is a major constituent of the extracellular matrices in which most tissues differentiate. It is also an essential component of many extracellular matrices in mature tissues. In some cases, hyaluronan is a major constituent. For example, in the vitreous of the human eye (0.1-0.4 mg/g wet weight), or in synovial joint fluid (3-4 mg/ml), or in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml), or in the pathological matrix that occludes the artery in coronary restenosis. In others, while representing less of the mass of the tissue, hyaluronan serves as an essential structural element in the matrix. For example, hyaluronan is present at ~1 mg/g wet weight in hyaline cartilages, enough to fill the tissue volume in the absence of other constituents. However, aggrecan, the large chondroitin sulfate proteoglycan, is present at a much higher concentration (25-50 mg/g wet weight), and hyaluronan retains aggrecan molecules in the matrix through specific protein-hyaluronan interactions which mask the hyaluronan backbone. Hyaluronan is less concentrated in the matrix of other connective tissues, such as those surrounding smooth muscle cells in the aorta and fibroblasts in the dermis of skin. Like cartilage however, hyaluronan forms a scaffold for binding large chondroitin sulfate proteoglycans in the matrices of these tissues (http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html).

The largest amount of hyaluronan (7-8 g per average adult human, ~50% of the total in the body) resides in skin tissue, where it is present in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue). Interestingly, while dermis consists primarily of extracellular matrix with a sparse population of cells, the epidermis is the reverse; the keratinocytes fill all but a few percent of the tissue. Thus, the actual concentrations of hyaluronan in the matrix around the cells in the epidermis (estimated to be 2-4 mg/ml) is an order of magnitude higher than in the dermis (estimated to be ~0.5 mg/ml). The matrix around keratinocytes, then, may have a hyaluronan concentration as high as that in umbilical cord (~4 mg/ml) considered to be the mammalian tissue with one of the highest concentrations. Interestingly, rooster comb, a specialized piece of skin, has even higher amounts of hyaluronan (up to 7.5 mg/ml). Several functions of hyaluronan have been assigned to HA, such as

stabilization of extracellular matrix, joint lubrication, water homeostasis, regulation of plasma protein distribution, barrier with the spread of infectious agents and macromolecules, and regulation of cellular activities by its interactions with cell surface receptor proteins (Martins *et al.*, 2003).

1.2.7 Cancer and Metastasis

Cancer is a genetic disease that usually arises from the accumulation of several mutations. Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation. Continuous division of these cells results in the formation and growth of tumors. A second important process that leads to cancer is that cancerous cells acquire the ability to leave the primary tumor and invade and form colonies at secondary sites. This process, known as metastasis, is caused by changes in the cell-substrate and cell-cell adhesion properties of tumor cells (Stubbs *et al.*, 2002).

Metastasis is defined as the formation of secondary tumor foci at a site discontinuous from the primary tumor. Metastases can form following invasion and penetration into adjacent tissues followed by dissemination of cells in the lymphatics, blood vasculature, coelomic cavities, or epithelial cavities. Metastatic cells arise within a population of neoplastic/tumorigenic cells as a result of genomic instability. This subset of cells has accumulated mutations in addition to those that have already rendered the cells tumorigenic. Metastasis-competent cells have evolved so that they detach and migrate away from the primary tumor. During transport, cells travel individually or as emboli composed of tumor cells (homotypic) or of tumor cells and host cells (heterotypic). At the secondary site, cells or emboli arrest either because of physical limitations (i.e., too large to traverse a lumen) or by binding to specific molecules in particular organs or tissues. Once there, tumor cells then proliferate either in the vasculature or in the surrounding tissue after extravasation. To form macroscopic, clinically detectable metastases (on the order of mm), cells recruit a vascular supply. To form clinically important metastases, cells must complete every step of this complex cascade and proliferate at the secondary site (Welch and Rinker-Schaeffer, 1999). Metastasis show in Figure 12.

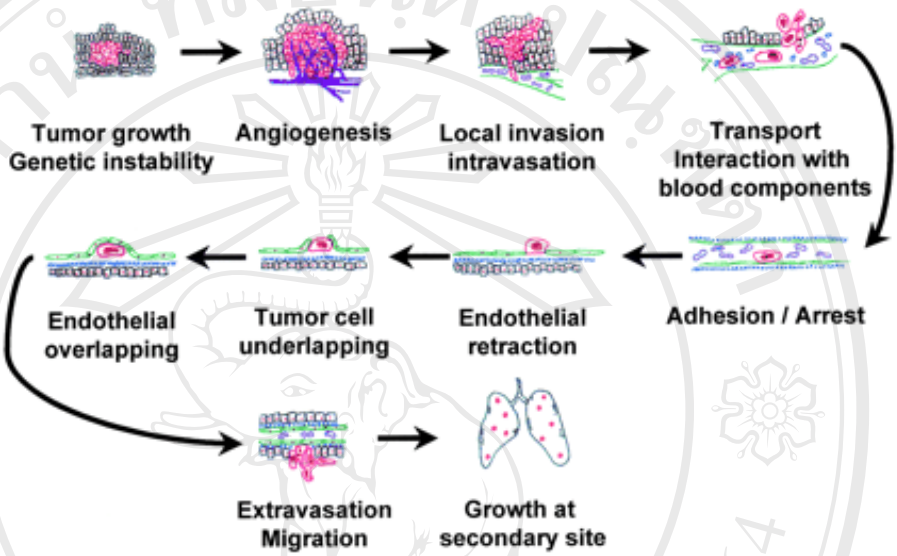

Figure 12. Pathogenesis of hematogenous metastasis (Hlatky *et al.*, 1994).

1.2.8 Role of Hyaluronan in Cancer Progression

The models of carcinogenes is describe cancer as a progression of genetic mutations in a tumor cell mass. These models have contributed to the discoveries of many tumor suppressor genes and potential oncogenes. Solid tumors, however, are more than clonal expansions of tumor cells; tumors are heterogeneous and have a complex structure. Furthermore, Bissell describes a tumor as a unique "*organ*" formed by "*tissues*" (Bissell and Radisky, 2001). The cells composing these tissues interact with each other and with other types of cells and exchange information through cell-cell interactions or through interactions with cytokines and the extracellular matrix (ECM). These combined interactions facilitate tumor progression. A number of authors have suggested that the ECM plays a significant role in tumor growth, progression, and metastatic development (Bissell and Radisky, 2001). Tumor invasion and metastatic development are mediated through the breakdown of the ECM. Disruption of the ECM promotes abnormal inter and/ or intra- cellular signaling leading to abnormal cell proliferation, cell growth, cytoskeleton reorganization, and alteration of other cell functions. HA is involved in many biological processes including malignant cell migration (Adamia *et al.*, 2005).

A large body of experimental evidence from animal models directly implicates hyaluronan in the progression of several tumor types (Kosaki *et al.*, 1999). Two major approaches have been used to probe the involvement of hyaluronan. First, it has been shown that overexpression of Has (hyaluronan synthases) promotes growth of fibrosarcoma and prostate carcinoma (Kosaki *et al.*, 1999; Liu *et al.*, 2001) and metastasis of mammary carcinoma (Yabushita *et al.*, 2005) *in vivo.* Second, perturbation of endogenous hyaluronan interactions inhibits growth, invasion, and metastasis in several tumor types *in vivo* (Peterson *et al.*, 2000). Several approaches have been used to manipulate hyaluronan interactions, the most common of which has been overexpression of soluble hyaladherins, *e.g.* soluble CD44 (Yu *et al.*, 1997), RHAMM , or hyaladherins from cartilage extracts (Liu *et al.*, 2001). Soluble hyaladherins act as an interactive sink for displacement of endogenous hyaluronan from its receptors, *e.g.* CD44, thus inhibiting putative downstream events. For example, overexpression of soluble CD44 in mammary carcinoma or melanoma cells inhibits tumor growth and metastasis, but these effects are not obtained if the soluble CD44 is mutated such that hyaluronan binding does not occur (Peterson *et al.*, 2000). Cellular effects of soluble CD44 include induction of G_1 arrest (Peterson

et al., 2000) or apoptosis (Yu and Stamenkovic, 1999) in the tumor cells and inhibition of MMP-mediated invasion. Administration of hyaluronan oligosaccharides also inhibits growth of several tumor types *in vivo*, including mammary and lung carcinomas and melanoma (Zeng *et al.*, 1998). Hyaluronan oligomers compete for endogenous polymeric hyaluronan-receptor interactions, resulting in monovalent rather than polyvalent interactions with receptors. Recent results have shown that hyaluronan oligomers also induce G_1 arrest or apoptosis in tumor cells (Auvinen *et al.*, 2000). Another consequence of treatment with soluble hyaladherins is the loss of hyaluronan-induced clustering of CD44 in the plasma membrane. Clustering of CD44 in the membrane leads to docking of gelatinase B (MMP-9) on the surface of mammary carcinoma and melanoma cells. This phenomenon results in promotion of tumor cell invasiveness and angiogenesis (Yu and Stamenkovic, 2000), both of which are important events in tumor progression. Overexpression of membrane-bound CD44 can also disrupt clustering (Yu and Stamenkovic, 1999), possibly explaining some of the apparently contradictory findings concerning the relationship of CD44 levels to tumorigenesis, *e.g.* inhibition (Schmits *et al.*, 1997) versus promotion of tumor progression by overexpression of intact CD44. Thus, there is likely a fine balance between the amount and organization of CD44 and its ability to respond to interaction with high molecular weight hyaluronan. Although it is clear that hyaluronan interactions directly influence various intracellular signaling pathways important for cell behavior, binding of hyaluronan to CD44 also leads to internalization and degradation (Toole *et al.*, 2002).

In this regard it is significant that some tumor cells exhibit elevated levels of hyaluronidase and the ability to internalize and degrade hyaluronan (Liu *et al.*, 1996). Thus penetration of hyaluronan-rich stroma (Yu *et al.*, 1997) or production of angiogenic breakdown products of hyaluronan may also promote tumor progression. Scheme of HA-CD44 signaling pathway show in Figure 13. Hyaluronan-RHAMM interactions have also been implicated in tumor cell behavior *in vitro* and *in vivo*. RHAMM is involved in the Ras and extracellular signal-regulated kinase signaling pathways and associates with the cytoskeleton (HA-RHAMM signaling pathway show in Figure14) (Turley *et al.*, 2002). Hyaluronan-RHAMM interactions induce transient phosphorylation signaling proteins in concert with turnover of focal adhesions in Ras-transformed cells, thus leading to initiation of locomotion (Chen *et al.*, 1995). Suppression of this interaction inhibits both cell locomotion and proliferation *in vitro* and leads to

inhibition of tumor growth *in vivo*, whereas overexpression of RHAMM leads to enhanced tumor growth and metastasis (Chen *et al.*, 1995; Turley *et al.*, 2002).

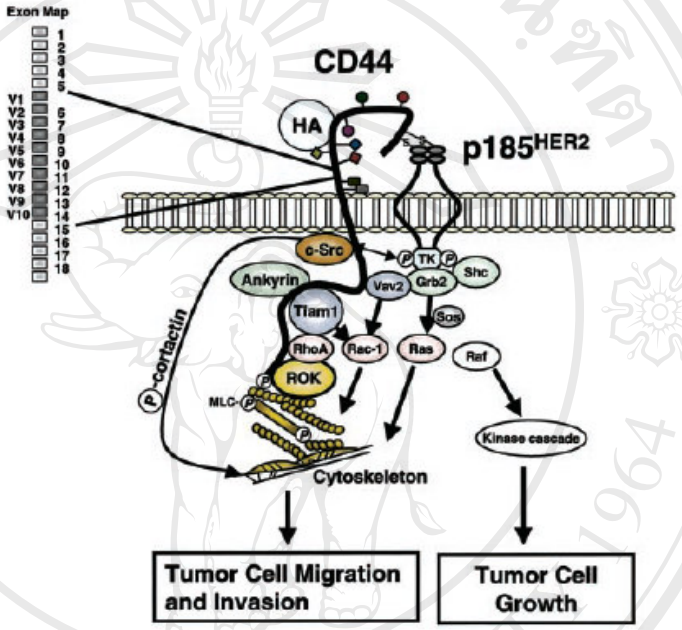

Figure 13. The model of hyaluronan-CD44 specific signaling pathways. Hyaluronan (HA) also promotes the association of CD44 forms with cytoskeletal proteins such as ankyrin and ERM proteins. Activation of these signaling pathways together leads to tumor behavior such as migration and invasion (Turley *et al.*, 2002).

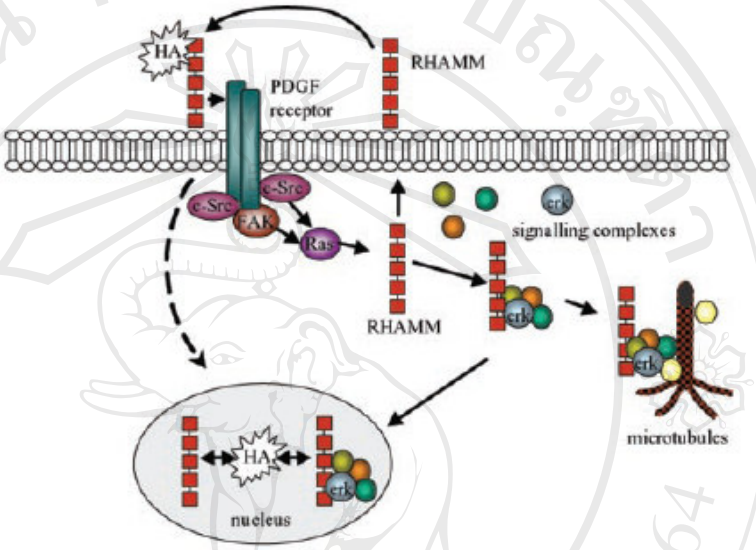

Figure 14. The model for hyaluronan-RHAMM mediated signaling pathways. RHAMM is hyaladherin that occurs in multiple subcellular compartments and that can also be exported to the extracellular matrix where it binds to the cell surface. Cell surface RHAMM-hyaluronan interactions regulate signaling through Ras and Src (Turley *et al.*, 2002).

1.2.9 Elevation of Hyaluronan in Cancer Disease.

Since hyaluronan is involved in the regulation of normal developmental processes such as proliferation and migration. Significantly increased levels of HA are often associated with certain types of human tumors, and the levels of HA in the serum of some cancer patients were also significantly elevated over those of normal individuals, its overproduction often correlates to pathologies that are characterized by inappropriate cell division and motility. Importantly, elevated levels of circulating hyaluronan occur in patients with colon, breast, bladder and prostate cancers, and hyaluronan deposits are increased within the primary tumors. Depending on the tumor, these hyaluronan deposits are associated with the carcinoma and/or with the tumor-associated stromal cells. These observations emphasize a potentially important link between hyaluronan accumulation and tumor progression in humans (http://www.glycoforum.gr.jp/science/hyaluronan/HA26/HA26E.html).

High hyaluronan levels are associated with many human cancers. Studies on histological sections from various tumors, using a specific hyaluronan affinity probe, have indicated that virtually all human epithelial tumors are surrounded by a connective tissue matrix (stroma) enriched in hyaluronan. Hyaluronan is thus a central component of the distinct stroma that surrounds and probably supports the tumor. The tumor extracellular matrix (ECM) is also enriched in hyaluronan-binding proteoglycans such as versican (Nara *et al.*, 1997).

Furthermore, some breast, stomach, and colon carcinomas show ectopic expression of hyaluronan associated with the malignant cells themselves, whereas the corresponding normal epithelia give virtually no signal for hyaluronan (Ropponen *et al.*, 1998; Auvinen *et al.*, 2000). Again, a high number of hyaluronan-positive cells predicts unfavorable outcome. For instance, the recurrence rate of colon carcinoma after an operation increases from 20 to 80% with increasing levels of hyaluronan associated with the carcinoma cells (Ropponen *et al.*, 1998). Likewise, elevated levels of hyaluronan and hyaluronidase in the urine form a clinically reliable marker for the presence and grade of bladder cancer (Lokeshwar *et al.*, 2000).

Several studies examining hyaluronan content in human prostate cancer samples have correlated the intensity of hyaluronan staining within the tumor-associated stroma to the severity of the cancer. Progression is associated with expansion of the glandular prostate carcinoma cells with a corresponding increase in the hyaluronan content of the tumor-associated stroma.

The hyaluronan in advanced primary tumors is easily extracted and is diverse in molecular weight, containing smaller hyaluronan fragments that facilitate angiogenesis of the primary tumor. These smaller hyaluronan fragments are produced by the action of tumor-associated hyaluronidase which has also been associated with prostate tumor progression. These fragments, along with other tumor related factors, contribute to increased vascularization of the tumor that accompanies tumor growth and progression (Figure 15).

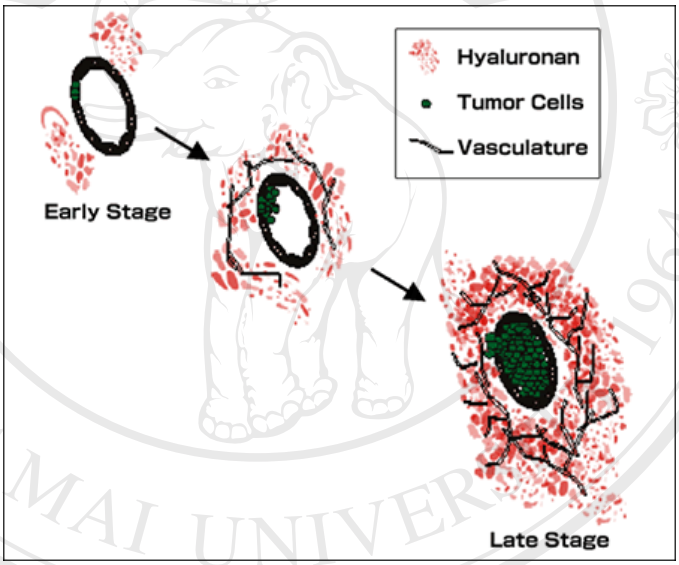

Figure 15. Elevated hyaluronan in primary prostate tumors. Prostate cancer progression is associated with elevations in hyaluronan. The diagram depicts progression within the primary tumor of prostate cancer.
(http://www.glycoforum.gr.jp/science/hyaluronan/HA26/HA26E.html)

1.2.10 Measurement of Hyaluronan

Around 1970 an increasing interest in the role of HA in cell biology (Toole *et al.*, 1977) and clinical practice (Butler *et al.*, 1970) stimulated researchers to develop new methods for its specific analysis. Microgram amounts of hyaluronan (HA) can be quantified by a variety of methods, the oldest and most widespread being colorimetry, using modifications of the carbazole method for the determination of hexuronic acid. In clinical analysis, HA is determined by electrophoresis on a cellulose acetate membrane It can also be determined by size-exclusion chromatography with UV photometric detection at a low wavelength (Jeanloz, 1960), but sensitivity and selectivity are insufficient for determinations in blood samples. In recent years, proteins with a specific affinity for HA have been utilized within rather complex methods which employ radioactive tracers together with hyaluronic acid binding protein (HABP), a part of the core protein of proteoglycan (Laurent *et al.*, 1996; Fraser *et al.*, 1997). Therefore measurement of HA is usually performed by a conventional radioimmunoassay (RIA) which was first described by Brandt et al. (Hardingham and Muir, 1972) and is still in use today with small modifications (Rossler, 1998).

The quantitation of HA in tissues and body fluids may provide a useful marker for diagnostic purposes. Several types of assays have been devised for measuring HA. However, these methods are often of limited usefulness as many of these assays require relatively large amounts of material whereas others are highly sensitive but require purification of the HA before assaying. RIA methods offer a high degree of sensitivity and do not require prior purification but need the use of radioactive tracers such as [^{3}H]-hyaluronate and, [^{125}I]-hyaluronectin and hence all the disadvantages of such a method (Rossler, 1998).

In 1998, Rossler devised a new test for HA. This assay was performed as an alternative to the conventional radiometric assay and to ELISA and employed the principle of time-resolved fluoroimmunoassays (Rossler, 1998). Recently, Martin and colleagues studied and developed method using a fluorescence-based assay for HA determination in serum of normal and cirrhotic patients (Martins *et al.*, 2003).

Hyaluronan has been reported to be a potential biomarker for liver, cancer and bone diseases. It can bind selectively to the proteins called HA-binding proteins (HABPs). In this study, the direct competitive ELISA based technique was carried out.

1.3 OBJECTIVES OF THIS STUDY

1. To prepare HABPs from chicken cartilage.
2. To develop fluorescence-ELISA technique and apply for determination HA by using FITC-HABP.
3. To apply fluorescence-ELISA based assay for HA determination in cancer serum.