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Review

Separation methods applicable to prostate cancer diagnosis and monitoring therapy

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Abstract

During the last decade, significant research has been conducted using prostate-specific antigen (PSA) in the basic and clinical sciences and many advances have occurred in the clinical use of PSA for detecting and monitoring prostate cancer (PCa). Separation methods including gel-permeation chromatography, isoelectric focusing, lectin-affinity chromatography, polyacrylamide gel electrophoresis and high-performance liquid chromatography have made significant contributions to the discovery and identification of different molecular forms of PSA. Furthermore, the measurement of free and total PSA has improved the ability of PSA to detect early PCa. However, unnecessary biopsies are still needed for men with slightly elevated PSA values. On the other hand, PSA is not adequate for staging newly diagnosed PCa and prognosticating the course in individual cases. The possible application of separation methods in the basic science of prostate cancer may be associated with identification of more cancer-specific forms of PSA and discoveries of other serum proteins useful not only for detecting, but also for staging and prognosticating PCa. Such novel markers might lead to a better understanding of PCa aggressiveness and to developments in the clinical field of treatment.

Keywords: Reviews; Prostate-specific antigen

Contents

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1. Introduction

Prostate cancer (PCa) is the most commonly detected malignancy in men and the second leading cause of cancer death in most Western societies [1]. This has made PCa a major concern for men and health organizations worldwide. In recent years, major efforts have been made to reduce the mortality rate from this disease. In short, this means early detection and local definitive therapy, since there is no curative systemic treatment for PCa.

Since its isolation and description in seminal fluid and prostatic tissue and ultimately in human serum two decades ago, prostate-specific antigen (PSA) has been the subject of intensive basic science and clinical investigations [2–5]. The introduction of PSA testing in 1986 revolutionized the management of patients with PCa. In less than 10 years, PSA has moved to the forefront of oncology testing. Prostatic acid phosphatase has no longer been used as a marker for prostate cancer for the last decade, because of its low sensitivity compared to PSA. Understanding of the relative organ specificity of PSA and the relationship of elevated serum PSA levels to prostate disease in general, coupled with the development of more refined biopsy techniques for histologic confirmation of diagnosis, realized the potential of PSA and increased the awareness of the clinical value of PSA testing. This awareness has resulted in a rapid expansion of the clinical applications of PSA testing. PSA has established itself as the most useful tumor marker for the early detection and staging of PCa as well as for monitoring the response to therapy [6–8]. Nevertheless, PSA has important limitations that prevent it from being the ideal tumor marker for the early detection of PCa. This is because PSA is not cancer-specific; as a result, serum PSA levels over the reference range are not pathognomonic for PCa and values within the reference range do not always indicate the absence of PCa. This lack of assay performance, most particularly limited specificity, has led researchers to investigate various methods for improving the reliability of the PSA test. PSA derivatives have been purported to provide increased specificity and thus decrease the frequency of unnecessary biopsies. Despite some enthusiasm for PSA velocity (the rate of change in PSA from serial measurements), PSA density (correction for prostate volume) and age-specific PSA cutoffs, these have not been accepted as reliable parameters by most clinicians because of overall lack of performance compared with PSA alone [9–15]. Recent studies have demonstrated that PSA is present in the serum in several different molecular forms and that the relative amounts of the various PSA isoforms may vary according to specific pathologic states present in the prostate gland [16–18]. Moreover, numerous clinical investigators have shown the benefits of using specific forms of PSA to enhance the clinical utility of PSA in the early detection of PCa [19–27]. Separation methods including gel-filtration chromatography, isoelectric focusing, lectin-affinity chromatography, polyacrylamide gel electrophoresis and high-performance liquid chromatography have contributed to the discovery and identification of different molecular forms of PSA. This paper presents our current understanding of PSA molecular forms, focusing on separation methods that have been applied to identify various forms of PSA, and attempts to predict the future application of these methods to the diagnosis and treatment of PCa.

2. PSA and separation methods

2.1. Biologic characteristics and basic structure of PSA

PSA is a glycoprotein consisting of a single polypeptide chain and a single N-linked sugar-moiety. PSA belongs to a human tissue kallikrein family, all coded by closely related genes on chromosome 19 [28–33]. Their gene products also are similar in three-dimensional structure. After some controversy in the past, a universal nomenclature has emerged; defining human kallikrein 1 (hK1) (tissue kallikrein; not found in the prostate), hK2 (human glandular kallikrein; detectable in serum and prostate), and hK3, which is PSA [18,34]. The transcription and expression of PSA are basically restricted to the epithelial cells of the prostate and periurethral glands and are dependent upon androgen mediation, although low-level synthesis of the protein has been described in other tissues [35,36]. PSA is translated as a proenzyme, pre-pro-PSA (261 amino acids).

Pre-pro-PSA has 24 additional residues that consist of the pre-region (17 residues), which is the signal peptide, and the propeptide (seven residues). The signal peptide directs the protein to the membrane of the endoplasmic reticulum (ER) membrane. In the ER, the prepeptide is removed and the resulting pro-PSA is transported within vesicles to the plasma membrane, where it is secreted into the lumina of the prostate ducts (Fig. 1). Recently, it was suggested that hK2 is responsible for cleaving of the propeptide to form extracellular mature PSA (237 amino acids), which is enzymatically active [18]. On the other hand, the sugar-chain structure of PSA is determined by a series of processing reactions catalyzed by Golgi glycosidases and glycosyl transferases [37]. PSA is present in high concentrations in the seminal fluid (0.5~5 mg/ml). The physiological role of PSA is thought to be cleavage of gel-forming proteins such as seminogelin and fibronectin and enhancement of sperm motility in human semen [38,39]. Normal and benign hyperplastic prostate tissue produces more PSA protein than malignant prostate tissue, which also has been confirmed at the mRNA level [4,40,41]. Therefore, PSA is not a traditional tumor marker that is produced in higher quantities by malignant cells. It is not exactly clear how PSA enters the circulation, migrating from the apical part of the epithelial cell into the bloodstream. It is postulated that abnormalities of prostate gland architecture resulting from disease, especially cancer, can lead to an increased diffusion of PSA into the stroma and ultimately into vessels. Periurethral glands probably do not contribute to serum PSA levels, but can be a major source of postprostatectomy urinary PSA [42].

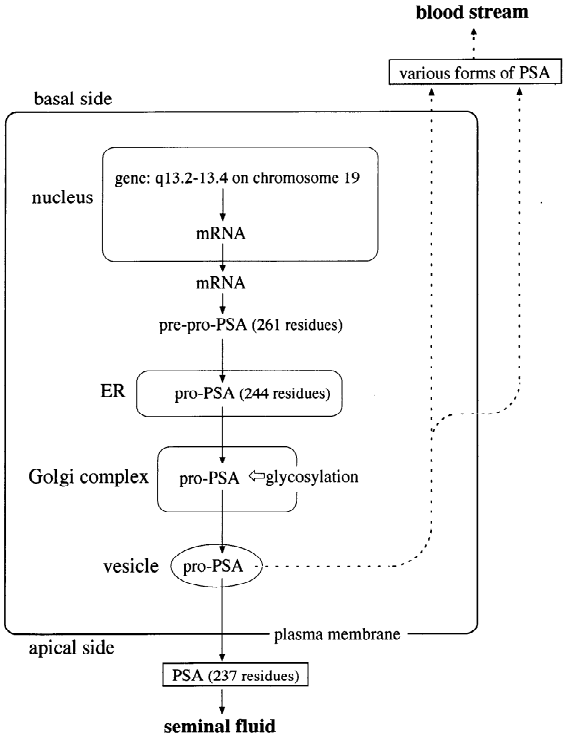

Fig. 1. Processing of PSA in epithelial cells of the prostate. ER, endoplasmic reticulum.

2.2. Molecular forms of PSA and separation methods

PSA is generally purified from either prostatic tissue or seminal plasma [4,43]. A typical purification scheme begins with ammonium sulfate fractionation, which is followed by anion-exchange chromatography, gel filtration, and preparative polyacrylamide gel electrophoresis [4,44]. Most of our knowledge regarding the biochemical properties of PSA stems from studies on the protein isolated from seminal plasma, in which concentrations are a million-fold higher than in serum and from which isolation is easier than from prostatic tissue.

PSA has an apparent molecular mass that ranges from 30 to $36 \cdot 10^3$ by gel filtration or gel electrophoresis [43]. A molecular mass of $26.08 \cdot 10^3$ corresponding to a sequence of 237 amino acid residues has been reported [31]. Furthermore the actual molecular mass of PSA, $28.43 \cdot 10^3$, was determined by Belanger et al., using ion spray mass spectrometry [45]. The difference between the results of calculation from the peptide moiety and by mass spectrometry is attributed to N-linked oligosaccharide. The greater apparent mass by gel filtration or electrophoresis is most likely due to the aberrant behavior of glycoproteins in these systems [18].

PSA purified from serum samples has a molecular mass of $90–100 \cdot 10^3$ by gel filtration, but is about $30–36 \cdot 10^3$ when separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [43]. The larger size was recently found to be attributable to the PSA complexed to alpha-1-anti-

chymotrypsin (ACT) [17]. Since its introduction into clinical practice, PSA in the serum has been measured largely as total PSA, which is composed of the bound (complexed) and unbound (uncomplexed or free) forms. When complexed, PSA is bound by endogenous protease inhibitors since it normally functions as a serine protease. The ACT irreversibly complexes the majority of circulating PSA, while alpha-2-macroglobulin (AMG) and others (protein C inhibitor, alpha1-antitrypsin) likely bind a smaller proportion [46]. PSA–ACT complex is thought to be enzymatically inactive [47], while some evidence suggests that PSA protease activity is not completely inactivated in PSA–AMG complex [48].

The fraction of free PSA in serum was found to be relatively lower in men with PCa compared to men without malignant disease [16–18]. This suggested that somewhere in the malignant epithelial prostatic cells, the metabolism of PSA was influenced by the disease. Enzymatically active PSA cannot exist in serum because of the abundant presence of protease inhibitors like ACT and AMG. Therefore, free PSA in serum was thought to be either pro-PSA (244 amino acid proform) or the 237-amino acid clipped form [18]. Hilz et al. has recently reported a novel procedure including size exclusion chromatography to purify PSA with a high yield from sera containing PSA at a concentration less than 10 ng/ml [49]. Using this method, they suggested that the major component of free PSA from serum is not in the clipped form nor pro-PSA. Further studies are needed to identify the molecular forms of free PSA in serum.

It has been suggested that ACT is produced within the cells, and more so in malignant prostate epithelial cells of poorer differentiation. At the same time, ACT was hardly produced in the nodules of benign prostatic hyperplasia (BPH) [50–53]. This is consistent with the clinical observations that the free PSA fraction in serum is lower in PCa. It was suggested by Ornstein et al. that intracellular PSA exists in the free form in both benign and malignant epithelium and that binding to ACT occurs exclusively outside of the cell. In their study, the techniques of laser capture microdissection and one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) were used [54].

Each PSA molecular form contains five immunoreactive, antibody binding sites (epitopes) [55]. Commercial PSA assays can detect total PSA, PSA–ACT complex and free PSA due to differences in antibody binding [18,55]. Discrepancies in total PSA measurement among commercial test systems were often observed at hospital laboratories until a few years ago. Studies using gel-filtration chromatography have illustrated that much of the quantitative differences are attributable to relative differences in recognition of the free and complexed form of PSA and calibration differences between assays (Fig. 2) [56–58]. Free PSA and PSA–ACT complex share three common epitopes. Immunological assays can distinguish between the two forms since another epitope is masked when ACT complexes PSA. Due to its large size, AMG encapsulates the PSA molecule completely and blocks all of the epitope sites [48]. As a result, conventional immunoassays have had much difficulty detecting PSA–AMG complex due to this lack of immunoreactivity. However, PSA in the PSA–AMG complex can be rendered immunoreactive by denaturation of AMG with sodium dodecyl sulfate [38] and detected in serum with high PSA levels by immunoblotting [57]. Zhang et al. have recently developed a quantitative assay based on the denaturation and measurement of the released PSA immunoreactivity by a conventional PSA assay [59]. In their preliminary study the proportion of PSA–AMG complex in serum was suggested to be lower in patients with PCa than in those with BPH.

2.3. Sugar-chain structure of PSA and separation methods

Although it has been suspected that O-linked oligosaccharides are present on PSA, recent studies have demonstrated that the protein contains only a single N-linked carbohydrate at asparagine 45 of the molecule [60]. This N-linked moiety comprises about 8% of the total mass of PSA. With regard to this moiety, a biantennary complex type with fucose linkages to the innermost *N*-acetylglucosamine (GlcNAc) was proposed as the major sugar-chain structure of PSA purified from seminal plasma by NMR spectroscopy (Fig. 3) [45]. However, the presence of four or five isoforms of PSA with different isoelectric points (pI) in BPH tissue and seminal plasma has been suggested by Wang et al.

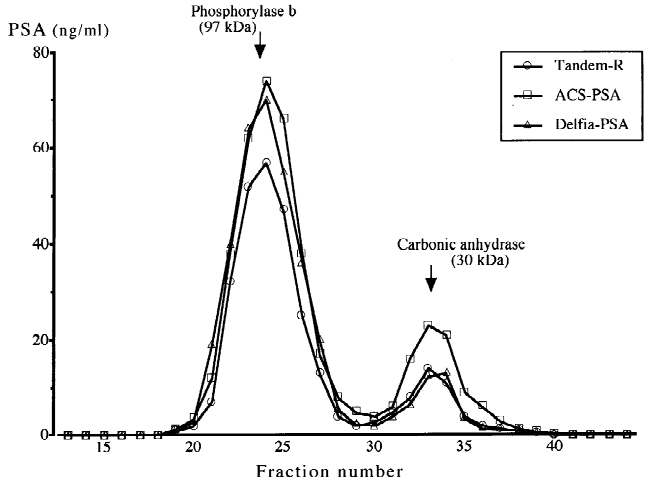

Fig. 2. Gel-filtration chromatographic pattern of serum from a patient with advanced prostate cancer, using a Sephacryl S-200 column. PSA values in each fraction were measured by three different PSA immunoassays (Tandem-R, ACS–PSA, Delfia–PSA). Using molecular mass markers, the main peak represents PSA complexed to alpha1-antichymotrypsin, while the smaller peak represents free PSA. Modified from Umeda et al. [58].

[44]. They also have shown that treatment with neuraminidase converts some isomers to isomers with a higher pI, suggesting that variations in the pI value reflect, at least in part, a difference in sialic acid content.

Alterations in sugar-chain structures of glycoproteins during oncogenesis have been reported in some human carcinomas [61–66]. In addition, abnormal or inadequate production of glycosyl- and fucosyl-transferases, which is responsible for the changes in sugar moiety, has also been demonstrated in malignant human tissues, such as hepatocellular carcinoma, choriocarcinoma, and pancreas carcinoma [67,68]. With regard to prostate carcinoma, Yoshida et al., using lectin affinity chromatography, has suggested qualitative differences in the sugar-chain structures of prostatic acid phosphatase between benign and malignant human prostate tissues [66]. However, few studies have been conducted on changes in the sugar-chain structures of PSA associated with malignant transformation. Although in 1989, Barak et al. reported significantly different PSA–Con A binding ratios in the sera of patients with PCa compared to those with BPH [69], other investigators have found considerable overlap between the two groups, suggesting that the measurement of this ratio lacks clinical utility [70–72]. PSA in the serum exists predominantly in a complex with ACT, which is itself extensively glycosylated; therefore, the lectin-binding properties determined in the studies using total serum PSA may reflect the sugar-chain structures not only of PSA, but also of ACT. In contrast, PSA observed in extracts of prostatic tissue, either benign or malignant, has been shown to be noncomplexed [44,73]. For that reason, we recently compared PSA derived from BPH tissue with that

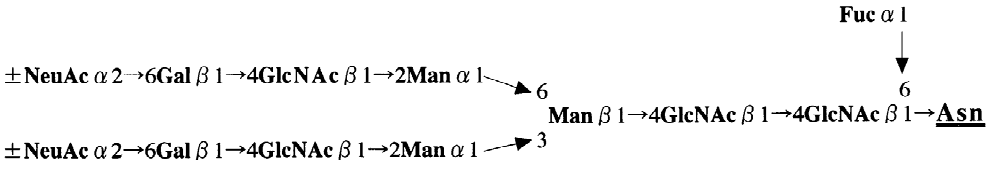

Fig. 3. Proposed major sugar-chain structure of PSA purified from seminal plasma by NMR spectroscopy. NeuAc, *N*-acetylneuraminic acid; Gal, galactose; Man, mannose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose.

derived from PCA tissue in terms of their lectin-binding characteristics to determine whether or not any structural alterations take place in the sugar-chains of PSA during oncogenesis of the human prostate [74].

2.4. Lectin affinity chromatography and other methods

Lectins have been used to detect glycoproteins that contain certain carbohydrate structures. The serial lectin affinity chromatographic technique using Con A (concanavalin A), PHA-E (phytohemagglutinin E), PHA-L (phytohemagglutinin L), PS (pea lectin) and WGA (wheat germ agglutinin) is useful in characterizing sugar-chain structures (Fig. 4) [75,76]. Con A strongly binds to the hybrid and high mannose types of sugar-chains. However, this binding does not occur if the biantennary sugar-chain structure undergoes modification through bisecting glucosaminylation, or if further branching leads to the formation of multiantennary structures. Binding specificities of the other lectins employed are PHA-E, which binds to a complex type containing a bisecting GlcNAc-beta (1→4) linkage; PHA-L, which binds to a multiantennary (triantennary or tetra-antennary) complex type with GlcNAc-beta (1→6) branches; PS, which binds to a biantennary complex type with fucose linkages to the innermost GlcNAc; and WGA, which binds to a hybrid type without fucose linkages to the innermost GlcNAc.

Possible sugar-chain structures separated by the method of serial lectin affinity chromatography are shown in Table 1 [75]. Using this method, we have shown that relative amounts of PSA with type I sugar chains (multiantennary complex type with

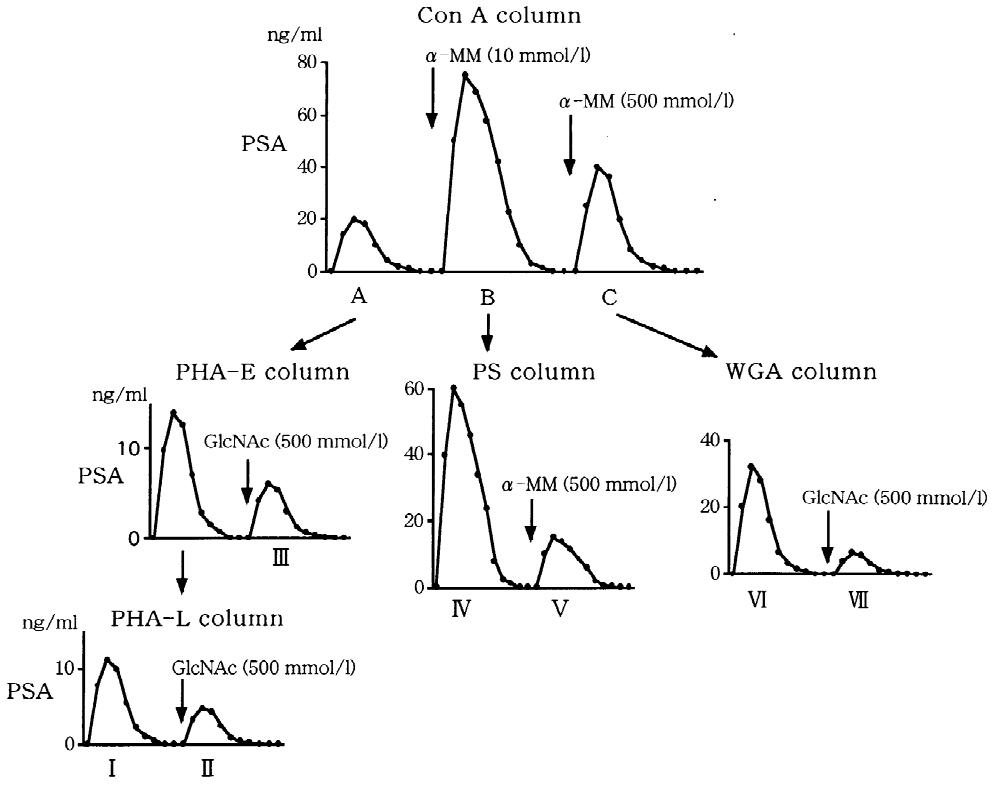

Fig. 4. Separation of glycoproteins into seven fractions by serial lectin affinity chromatography. Fractions A, B and C obtained on the Con A column are subsequently applied to PHA-E, PS and WGA columns, respectively. The unbound fraction on the PHA-E column is further applied to the PHA-L column. α-MM, α-methyl-D-mannoside; GlcNAc, *N*-acetylglucosamine. Arrows indicate the starting points of elution.

Table 1
Possible sugar-chain structures of given fractions of glycoproteins isolated by serial lectin affinity chromatography

Type	Characteristics of sugar chains	Proposed structure
I	Multiantennary complex type with branched GlcNAc(β1→4)Man	±NeuAc α2→6Gal β1→4GlcNAc β1→4 ±NeuAc α2→6Gal β1→4GlcNAc β1→2 Man α1→6 ±NeuAc α2→6Gal β1→4GlcNAc β1→4 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β1→2 Man α1→3
II	Multiantennary complex type with branched GlcNAc(β1→6)Man	±NeuAc α2→6Gal β1→4GlcNAc β1→6 ±NeuAc α2→6Gal β1→4GlcNAc β1→2 Man α1→6 ±NeuAc α2→6Gal β1→4GlcNAc β1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β1→2 Man α1→3
III	Bisecting GlcNAc(β1→4) containing complex type	±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→3 (4 ↑ GlcNAc β1)
IV	Biantennary complex type without fucose linkages to the innermost GlcNAc	±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→3
V	Biantennary complex type with fucose linkages to the innermost GlcNAc	Fuc α1↓6 ±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β1→2Man α1→3
VI	① High mannose type or ② Hybrid type with fucose linkages to the innermost GlcNAc	Man α1→6 Man α1→3 Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn Man α1→3 Fuc α1↓6 Man α1→6 Man α1→3 Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β→2Man α1→3
VII	Hybrid type without fucose linkages to the innermost GlcNAc	Man α1→6 Man α1→3 Man α1→6 Man β1→4GlcNAc β1→4GlcNAc β1→Asn ±NeuAc α2→6Gal β1→4GlcNAc β→2Man α1→3

New AC, *N*-acetylneurominec acid; gal, galactose; glc NAc, *N*-acetylglucosamine; FUC, fucose.

branched GlcNAc-beta (1→4) Man) are significantly greater for PCa-derived PSA than BPH. Furthermore, the sum of type IV and V (biantennary complex type without/with fucose linkages to the innermost GlcNAc) was significantly decreased in PCA compared to BPH [74]. These findings suggest that *N*-acetylglucosaminyl transferase IV (GnT-IV), which adds GlcNAc to sugar chains by beta (1→4) binding and converts biantennary to multiantennary types, is expressed at higher levels in PCA (Fig. 5).

Several studies have demonstrated that the activity of GnT-V, which adds GlcNAc to the trimannosyl

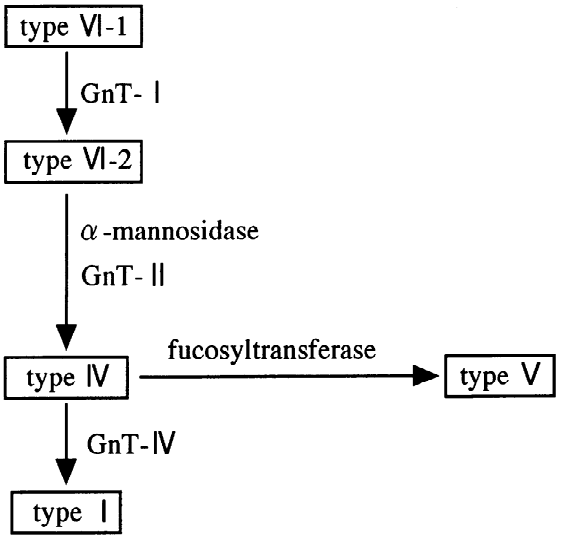

Fig. 5. The relationship between *N*-acetylglucosaminyl transferase in the Golgi complex and the four types of N-linked sugar-chain structures of PSA derived from prostate tissue. Of the seven sugar-chain types fractionated by serial lectin affinity chromatography, types II, III and VII were undetectable in both BPH and PCa. type I, multiantennary complex type with branched GlcNAc (β 1→4) Man; type IV, biantennary complex type without fucose linkages to the innermost GlcNAc; type V, biantennary complex type with fucose linkages to the innermost GlcNAc; type VI-1, high mannose type; type VI-2, hybrid type with fucose linkages to the innermost GlcNAc; GnT, *N*-acetylglucosaminyl transferase.

core by beta (1→6) binding, is increased in malignant cells and correlates in many instances with the metastatic potential of cells [64,68,78–83]. Thus, similar to GnT-V in those instances, the increased activity of GnT-IV in prostate carcinoma might have some effect on the cellular phenotypes, including increased adhesion to vascular endothelial cells, increased cellular motility and invasion of the extracellular matrix, and increased cellular response to autocrine growth stimulation.

Prakash et al. recently investigated the N-linked structure on PSA made by a cell line derived from metastatic prostate tissue, using fluorophore-assisted carbohydrate electrophoresis [77]. They observed that unlike normal PSA from seminal plasma, which bears only biantennary oligosaccharides, PSA from a metastatic cell line had a mixture of biantennary, triantennary and also tetra-antennary oligosaccharides. These findings are concordant with ours.

Although the relationship between the sugar-chain structures and isoelectric points of PSA has not yet been clarified, it has been suggested in our laboratory that PSA with type I sugar chains has a relatively lower pI.

2.5. Pro-PSA and B-PSA

In 1997, Mikolajczyk et al. examined the nature of the free PSA form detected in serum using hydrophobic interaction chromatography–high-performance liquid chromatography (HIC–HPLC) [84] and showed that the precursor form of PSA, pro-PSA, is a component of free PSA in the serum of PCa patients. Recently they have investigated tissue distribution and reported that pro-PSA is differentially elevated in peripheral zone cancer (PZ-C), but is largely undetectable in benign tissue from the transition zone (TZ) [85]. The pro-PSA is shown to contain pro leader peptides of two or four amino acids. These findings suggest that the ''Pro-PSA'' in serum may represent a more cancer-specific form of PSA. On the other hand, a form of free PSA more associated with BPH than PCa has also been reported by Mikolajczyk et al. using HIC–HPLC [85]. They have identified a specific molecular form of clipped free PSA, called B-PSA, that is increased in the prostatic TZ of patients exhibiting nodular BPH. B-PSA was purified and found to contain a characteristic clip between Lys 182 and Ser 183. At present, quantitative evaluation of different molecular forms of free PSA in serum is generally difficult. Furthermore, it has been suggested that mature inactive PSA, various forms of clipped inactivated PSA and smaller PSA fragments are also included in the free PSA in serum [49,86,87]. However, if the serum levels of specific forms of free PSA reflect the pathological status in the prostate, immunoassays that specifically measure Pro-PSA or BPSA might improve the ability of currently available PSA tests to discriminate patients with early PCa from those with BPH.

3. Future directions of PSA research and other markers for prostate cancer

Significant research has been conducted using PSA in the basic and clinical science for the last

decade. There is no question that serum PSA is a powerful tool for the early detection of PCa. Widespread use of PSA screening of asymptomatic men has resulted in a dramatic increase in the diagnosis and treatment of PCa [88]. There can also be no question that serum PSA is useful for monitoring clinical recurrence following treatment with curative intent (radical prostatectomy or radiation therapy) or detecting disease progression after hormonal manipulation. Nevertheless, PSA is not adequate for staging newly diagnosed PCa and prognosticating the course of the disease in individual patients. In addition, unnecessary biopsies are still needed for men with slightly elevated PSA values, though the measurement of free and total PSA has provided a significant improvement with respect to the specificity of PSA.

Among the various forms of PSA, the multiplicity of free PSA molecular forms is recently recognized and gaining much attention. To detect structural alterations associated with malignant transformation, free PSA is possibly a better object compared to complexed PSA. Specific forms of free PSA, such as Pro-PSA and PSA with a multiantennary complex type sugar-chain with GlcNAc-beta (1→4) branches, have been shown to be differentially elevated in PCa tissue. Although the quantity of these PSA forms in serum is, at present, unknown, if their measurement becomes possible, it might lead to a more cancer-specific marker for PCa. In the near future, studies will focus on investigating differences in structures of PSA and other proteins between benign and malignant prostate tissues, using various separation methods. Moreover the differences found in tissues might be applied to differences in serum PSA, leading to the development of a more useful marker.

Though it has been suggested that the measurement of PSA–AMG complex improves the cancer-specificity of the PSA test, this needs to be confirmed in a large screening setting. Furthermore the method reported by Zhang et al. [59] is technically complicated and should be automated to become clinically useful.

In addition, future studies will focus on further characterizing other markers for PCa. Presently studies are performed to determine the usefulness of hK2 [89–92], prostate specific membrane antigen (PSMA) [93–95], vascular endothelial growth factor [96], transforming growth factor-beta1 [97], caveolin-1 [98] and others. Except for hK2 and PSMA, these compounds have recently emerged as potentially clinically useful markers.

We are now beginning to adopt a new approach to PCa, including chemoprevention and gene therapy. These fields cannot develop without a complementary set of markers indicating disease status and prognosticators. Not only novel serum markers more specific than PSA for detecting PCa, but also novel markers useful for staging and prognosticating PCa are expected to emerge in the near future.

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