Disease Markers 25 (2008) 49–58
IOS Press

Fibronectin pattern in benign hyperplasia and cancer of the prostate

Miroslava M. Janković* and Maja M. Kosanović
Institute for the Application of Nuclear Energy-INEP, University of Belgrade, Belgrade, Serbia

Abstract. Fibronectin (FN) is a multifunctional glycoprotein involved in cell-matrix interactions. It exhibits a complex pattern of forms differing in respect to aminoacid and oligosaccharide composition. In this study we examined glycobiochemical and functional properties of the FN in benign prostatic hyperplasia (BPH) and prostatic cancer (PCa), attempting to resolve disease-related differences. Two BPH sera pools and three PCa sera pools were used as the FN source. The affinity-purified molecule was characterized by SDS-PAGE, immuno- and lectin blot, lectin-affinity chromatography and adhesion assay. BPH FN existed as intact molecule, giving the main immunoreactive band at 220 kDa. In contrast, PCa FN comprised three main immunoreactive fragments of 140, 110 and 90 kDa. As for glycosylation the ratio of altogether lectin-reactive PCa FN was different from that of BPH FN manifested as a decrease of Con A- and an increase of LCA-reactive moieties. Fibroblasts adhered to both FN preparations in a concentration dependent manner, but with a significantly lower efficiency to PCa FN. The results obtained showing distinct structural characteristics of PCa FN compared to BPH FN could be important for modulation of its ligand and recognition properties expressed as gain or loss of functions or as specific markers of its origin.

Keywords: Cancer marker, molecular forms, glycosylation, biological activity

1. Introduction

Prostate health is of special medical importance. Prostate cancer (PCa) is the second most common cause of death from cancers in men in western countries. Approximately 218 900 new cases and 27 000 deaths from this malignancy were expected in during 2007 in the US alone [1]. In the EU, PCa accounts for 11% of total cancers in men and 9% of total cancer deaths [2]. On the other hand, benign prostatic hyperplasia (BPH) is also highly prevalent in older men, i.e. after 60 years of age, 60% have histological alterations and 40% obstructive and irritative symptoms [3]. The majority of new PCa cases are diagnosed as localized disease. The tumor grows slowly and its progression to locally advanced and metastatic stages varies widely and may take years [4]. As the consequence of prostate enlargement BPH progression leads to lower urinary tract symptoms and related outcomes, necessitating prostate surgery [5]. Considerable efforts have been made to improve the diagnosis and monitoring of both BPH and PCa, especially their differential diagnosis. Although recent investigations have yielded several candidates, no markers for BPH are accepted for use in clinical practice [6]. As for PCa, it is accompanied with marked alterations in the expression of a number of different molecules.

They comprise both biochemical and genetic markers, some of which are already used in everyday laboratory practice for diagnostic and prognostic purposes [7, 8]. Examinations of the structural properties of cancer biomarkers, including those related to prostate cancer are a focus of many current investigations [7–10]. On the one hand, they seem to be a promising adjunct for improving marker potential of molecules the concentrations of which are found in overlapping ranges in benign and malignant conditions. On the other hand, gaining more insight into the molecular structure of cancer biomarkers can be important for understanding the basis of tumor growth and progression and possibilities for its control and modification.

*Corresponding author: Miroslava Janković, INEP, Banatska 31b, 11080 Zemun-Belgrade, Serbia. Tel.: +381 11 199 949; Fax: +381 11 2682 743; E-mail: miraj@inep.co.yu.

ISSN 0278-0240/08/$17.00

In this study we examined the glycobiochemical and functional properties of the fibronectin (FN) molecule in benign prostatic hyperplasia and cancer. Fibronectin is a modular multifunctional molecule involved in processes associated with cell-matrix interactions [11–14]. It exists in two main forms: a cellular form made by different cell types and a plasma form synthesized by hepatocytes. Fibronectin is a glycoprotein consisting of two similar subunits of 220 kD, linked by two disulphide bonds. Each subunit has a mosaic structure composed of three types of individual domains, but extradomains can be also included. Thus, depending on the source, FN exhibits a distinct and complex pattern of molecular forms differing in aminoacid and oligosaccharide composition. In humans, there are potentially twenty forms of FN [11–14].

This glycoprotein is expressed highly in inflamed tissue and may be an acute phase reactant [15,16]. Its expression is also altered during neoplastic transformation [17–20]. Thus, elevated levels of plasma or cellular FN and its fragmentation have been documented during wound healing, arthritis, bladder cancer and gastrointestinal, head and neck cancers [21–25]. As for prostate cancer, there is a limited number of experimental data indicating intensive expression in tissue as well as changes in serum concentration in cancer patients, but without information about its glycobiochemical properties [26–28]. Since, plasma FN and its fragments have been shown to exhibit specific activity towards prostate tissue i.e. a malignant prostate cell-line, in terms of stimulation or inhibition of tumor activity, gaining more insight into its structure could be of special biomedical importance [29,30].

We isolated FN from BPH and PCa serum sources and the affinity-purified products were characterized using immuno- and lectin-based techniques to resolve any disease-related structural and functional differences. The results obtained indicated distinct microheterogeneity of FN, which may have both functional and clinical relevance in relation to prostate physiology.

2. Materials and methods

2.1. Materials

Monoclonal antibody to plasma FN, clone P1H11 was purchased from R&D Systems (Minneapolis, USA) and monoclonal anti-cellular FN antibody 1940 (EDA specific) was from Chemicon International (Temecula, CA, USA). Bovine serum albumin (BSA), acrylamide, N, N'-bis-acrylamide, Coomassie brilliant blue (CBB) and mannose (Man) were from Sigma (St. Louis, USA). Lactose and gelatin were from ICN Biochemicals (Cleveland, Ohio, USA). Sepharose 4B and Sephadex G-75 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Affinity columns with immobilized plant lectins, Vectastain Elite ABC kit, biotinylated anti-mouse IgG, biotinylated lectins and 3, 3'-diaminobenzidine (DAB) substrate kit for peroxidase were from Vector Laboratories (Burlingame, USA). Microtest III cell culture plates of 96 wells were from Falcon, USA. Molecular mass markers Roti-Mark and Roti Black silver staining kit were purchased from Carl Roth GmbH+Co. (KG, Germany). CNBr-activated Sepharose was from Amersham Biosciences (Uppsala, Sweden). Radioiodine (^{125}I) was from the Institute of Isotopes Co., Ltd., Budapest, Hungary. PSA concentration was determined using IRMA-PSA, a solid phase two-site immunoradiometric assay (INEP, Serbia), while FN concentration was measured with a QuantiMatrix Human Fibronectin ELISA Kit (Chemicon International, Temecula, CA, USA). Immobilion-P, polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, USA). All other chemicals were reagent grade.

2.2. Serum samples

This study was carried out, according to local ethical standards, on archive specimens of sera from BPH and PCa patients seen at the Clinical Centre, Pozarevac, Serbia and INEP-Zemun, Serbia for PSA determination as part of follow up. Diagnoses were confirmed using clinically established protocols based on PSA level, physical examination, ultrasound, histomorphometry and biopsy. Pools were formed randomly with no respect to age and patient treatment. Thus, 18 BPH sera samples were randomly divided into two groups and two pools was prepared (pool I and pool II), each consisting of 9 individual samples. PSA concentrations ranged from 0.5–3.6 μg/L for pool I and 0.5–3.4 μg/L for pool II. Samples of PCa sera were from patients diagnosed with locally advanced and advanced cancers. The PCa sera samples were taken from patients at the time when their PSA levels began to rise (after prostate cancer had been diagnosed and suitable treatment initiated) indicating disease progression. The 21 serum samples were randomly allotted to three groups and three pools was prepared (pool III, pool IV and pool V), each consisting of 7 individual samples. PSA concentrations ranged from 106–986 μg/L, 120–180 μg/L

and 130–468 μg/L for pools III, IV and V respectively. The pools were used immediately or stored in aliquots at -20°C until processed.

2.3. Fibronectin isolation

Fibronectin was isolated from BPH and PCa sera by affinity chromatography on a gelatin-Sepharose 4B column according to Ruoslahti et al. [31]. Gelatin was bound to CNBr-activated Sepharose according to the manufacturer's instructions. A serum pool was placed on the gelatin-Sepharose column (7 mL), equilibrated with 0.05 M PBS, pH 7.2 at room temperature and allowed to react for 3 hours. The column was washed with equilibration buffer, 0.05 M PBS, pH 7.2, and subsequently with 1M NaCl until the absorbance baseline was reached. Fibronectin was then eluted with 4 M urea at a flow rate of 1 mL/min. Fractions (3 mL) were collected and the elution was monitored by measuring the optical density at 280 nm. Fractions of maxima were pooled, extensively dialyzed against 0.05M PBS, pH 7.2 and concentrated by ultrafiltration.

2.4. SDS-PAGE

SDS-PAGE was performed on 6% separating gel and 3.75% stacking gel [32] and gel was stained using the Roti Black silver staining kit according to the manufacturers' instructions. The gel was calibrated with Roti-Mark molecular mass markers.

2.5. Immuno- and lectin-blotting

Proteins were transferred on to polyvinylidene fluoride (PVDF) membrane by semi-dry blotting using a Multiphor II Nova Blot Unit (Pharmacia LKB, Upsala, Sweden). The conditions were: transfer buffer: 25 mM TRIS, 192 mM glycine, 20% methanol pH 8.3; 1.2 mA/cm^2 for 1 hour. PVDF membrane was blocked with 1% casein in 0.05M PBS, pH 7.2, overnight at 4°C.

For immunoblotting, the membrane was incubated with monoclonal anti plasma-FN Ab or monoclonal anti cellular-FN Ab (both at 0.5 μg/mL in 1% casein 0.05 PBS, pH 7.2) overnight at 4°C, washed three times in 0.05% Tween in 0.05M PBS, pH 7.2, and then incubated with biotinylated anti-mouse IgG Ab for 30 min at room temperature (RT). After repeating the washing procedure, the membrane was incubated for 30 min at RT with a mixture of avidin and biotinylated horse radish peroxidase (HRPO) from Elite Vectastain ABC kit (prepared according to the manufacturer's instructions). The membrane was then washed again three times and incubated with substrate solution containing hydrogen peroxide solution and 3, 3'-diaminobenzidine (DAB) (prepared from the DAB substrate kit, according to the manufacturer's instructions), until the bands appeared.

For lectin blot, membranes were incubated with biotinylated lectins (10 μg/mL) for 1 hour at RT. After washing three times in 0.05% Tween in 0.05 M PBS, pH 7.2, a mixture of avidin and biotinylated horse radish peroxidase (HRPO) from Elite Vectastain ABC kit (prepared according to the manufacturer's instructions) was added and the whole incubated for 30 min at RT. The membrane was then washed again three times and incubated with substrate solution containing hydrogen peroxide solution and DAB (prepared as described above), until the bands appeared.

The corresponding negative controls (omitting the primary antibody/binding in the presence of lectin-competing sugars) were also performed and gave no visible reactions.

2.6. Iodination

Isolated FN from BPH pool I (BPH FN) and PCa pool III (PCa FN) were labeled with ^{125}I, using the chloramine-T method [33]. Labeled FN was separated from free iodine on a Sephadex G-75 column, equilibrated and eluted with 0.05 M PBS, pH 7.2 containing 0.05% bovine serum albumin. The specific activity of the iodinated preparations was 0.1 mCi/μg for ^{125}I-BPH FN and 0.2 mCi/μg for ^{125}I-PCa FN.

2.7. Lectin-affinity chromatography

Affinity chromatography of ^{125}I-FN was performed on columns of the following immobilized plant lectins: SNA (*Sambucus nigra* agglutinin), RCA I (*Ricinus communis* agglutinin I), Con-A (lectin from *Canavalia ensiformis*) and LCA (*Lens culinaris* agglutinin). The bed volume was 5 mL for Con-A and RCA-I, 3 mL for LCA and 2 mL for SNA columns. ^{125}I-FN (0.4 mL; $\sim$1000000 cpm) was loaded on to each of the columns. A common chromatographic scheme was applied to all columns, according to the manufacturer's instructions. After 3 hours incubation at room temperature, the fractions (1 mL for 5 mL columns or 0.5 mL for 2 mL and 3 mL columns) were collected. The unbound and the retarded fractions from SNA and RCA I columns were eluted with binding buffer (0.05 M PBS pH 7.2

for SNA and RCA I and 0.1 M acetate buffer pH 6.0, supplemented with 100 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ for Con-A and LCA columns). The bound fractions were specifically eluted by addition of competitive sugars: 0.1 M mannose (for Con-A and LCA) and 0.1 M lactose (for SNA and RCA). Finally, the tightly bound fraction was eluted with low pH buffers: 0.1 M glycin-HCl pH 3.0 (for RCA), 0.2 M acetic acid (for LCA) or 0.3 M lactose in 0.2 M acetic acid (for SNA). The Con-A column was finally eluted with 0.1 M borate buffer, pH 6.5. Elution of ^{125}I-FN was monitored by measuring the radioactivity of each fraction.

2.8. Fibroblast adhesion assay

COS-7 fibroblasts were grown in Dulbecco's minimal essential (DME) medium supplemented with 10% heat-inactivated fetal bovine serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin [34,35].

The adhesion assay was performed according to Hyytiainen and Keski-Oja [36]. Cell culture plates of 96 wells were coated with serial dilutions of a) BPH FN/PCa FN (isolated from BPH pool I (BPH FN) and PCa pool III (PCa FN) (starting concentration 26.5 μg/mL) or b) monoclonal anti- FN Ab (0.1 μg/50 μL per well) for 1 hour at RT.

a) FN coated wells: after washing three times with 200 μL 0.05 M PBS, pH 7.2, 100 μL 1% BSA (heat treated at 70°C) was added and incubated for 1 hour at RT to block non-specific binding. The washing procedure was repeated and the cell suspension was added to FN-coated wells (5×10^4 fibroblast cells in 50 μL FCS free DME medium) and incubated for 1 hour at 37 °C. Non attached cells were removed by gentle washing three times with 200 μL 0.05 M PBS, pH 7.2. Cells were then fixed with 4% paraformaldehyde for 10 min, washed and stained with 0.1% Coomassie brilliant blue in 30% methanol and 10% acetic acid. The excess of color was removed with 30% methanol and 10% acetic acid and the cells were finally washed with 0.05 M PBS, pH 7.2. The cells were lysed with 1% SDS and OD at 620 nm was measured using Microplate Reader LKB5060-006 (LKB, Austria).

b) Antibody coated wells: after washing three times with 200 μL 0.05 M PBS, pH 7.2, 100 μL 1% BSA (heat treated at 70°C) was added and incubated for 1 hour at RT to block non-specific binding. The washing procedure was repeated and then BPH and PCa pooled sera (FN concentration range: 0.4–162.0 μg/mL) was added and incubated for 1 hour at 37 °C. After another cycle of washing the cell suspension was added (5×10^4 fibroblast cells in 50 μL FCS free DME medium) and incubated for 1 hour at 37 °C. Non attached cells were removed by gentle washing three times with 200 μL 0.05 M PBS, pH 7.2. Cells were then fixed with 4% paraformaldehyde for 10 min, washed and stained with 0.1% Coomassie brilliant blue in 30% methanol and 10% acetic acid. The excess of color was removed with 30% methanol and 10% acetic acid and the cells were finally washed with 0.05 M PBS, pH 7.2. The cells were lysed with 1% SDS and OD at 620 nm was measured using the Microplate Reader LKB5060-006 (LKB, Austria).

All samples were tested in duplicate, and the results are presented as the mean value with error bars representing the standard error. Each assay was performed three times.

3. Results

3.1. Molecular forms of BPH FN and PCa FN

Representative SDS-PAGE and immunoblots of affinity purified BPH FN and PCa FN are shown in Fig. 1(A, B). BPH FN existed predominantly as an intact molecule, which, under reducing conditions, gave a main diffuse band at 220 kDa corresponding to the molecular mass of the FN subunit (Fig. 1.A.2, BPH FN). It was immunoreactive with monoclonal anti-FN (Fig. 1B, BPH FN).

Under non-reducing conditions the PCa FN preparation gave one higher molecular mass immunoreactive band at the interface of the stacking and separating gel, possibly corresponding to FN multimers (Fig. 1.A.2, PCa FN and Fig. 1B, PCa FN). Under reducing conditions, in contrast to BPH FN, three bands corresponding to 140, 110 and 90 kDa were dominant (Fig. 1.A.2, PCa FN) and their identities as FN fragments were confirmed by immunoblot analysis (Fig. 1B, PCa FN). Both preparations contained low molecular mass bands possibly corresponding to artifactual fragmentation, which may not be avoidable during the experimental procedures. In addition, none of the isolated BPH FN and PCa FN preparations were immunoreactive with monoclonal anti-EDA+ antibody specific for cellular FN (data not shown). The results presented are related to pools of BPH sera (pool I, $n = 9$, PSA range 0.5–3.6 μg/L) and PCa sera (pool III $n = 7$, PSA range 106–986 μg/L).

Although, total binding and yield were different, comparable results were obtained for the other indepen-

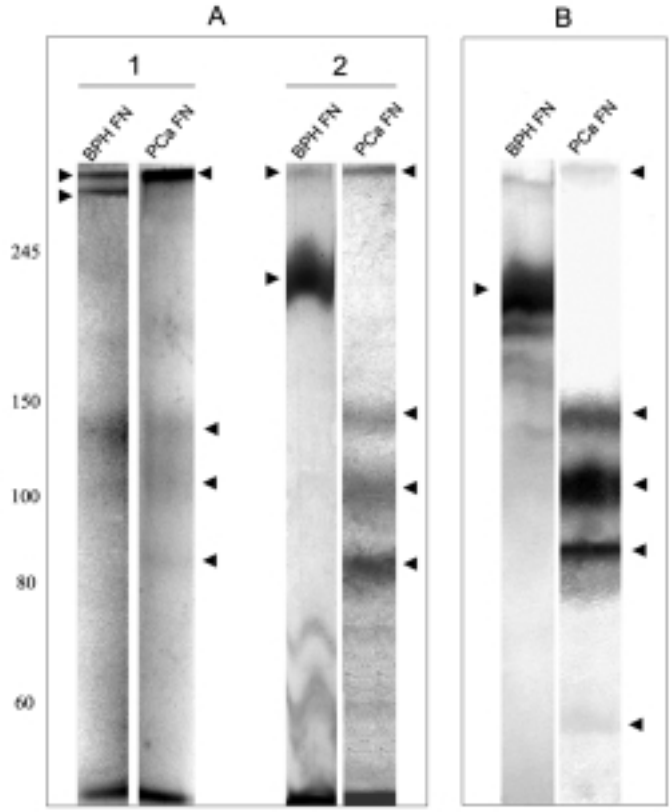

Fig. 1. SDS-PAGE and immunoblot of affinity-purified FN preparations. Fibronectin preparations, isolated from pools of BPH (pool I, $n = 9$, PSA range 0.5–3.6 μg/L) and PCa (pool III, $n = 7$, PSA range 106–986 μg/L) sera, by gelatin-affinity chromatography, were resolved on 6% separating and 3.75% stacking gel, and silver stained (A) or immunoblotted (B). Panel A (silver staining): BPH FN and PCa FN subjected to SDS-PAGE, under non-reducing (1) and reducing (2) conditions. Panel B (immunoblotting): after SDS-PAGE, under reducing conditions, proteins were transferred onto membrane and probed with monoclonal anti-FN antibody clone P1H11. Bound antibody was visualized using a mixture of avidin and biotinylated-HRPO followed by addition of H_2O_2/3, 3'-diaminobenzidine. Numbers indicate molecular mass (kDa).

dently examined pools of PCa sera (pool IV, $n = 7$, PSA range 120–180 μg/L, and pool V, $n = 7$, PSA range 130–468 μg/L) and BPH (pool II, $n = 9$, PSA range 0.5–3.4 μg/L). This indicates that the different qualitative pattern of FN-immunoreactive molecular forms was not due to sample to sample variations.

3.2. Glycosylation of BPH FN and PCa FN

The results of carbohydrate composition analysis of BPH FN and PCa FN under non-reducing and reducing condition using lectin blots are shown in Fig. 2A and Fig. 2B, 2C respectively. The following lectins were used: Concanavalin A, Con A (specific for the mannosyl core of high mannose-, hybrid- and biantennary complex-type of N-glycans), *Sambucus nigra* agglutinin, SNA (specific for sialic acid α2,6 linked Gal/GalNAc), *Ricinus communis* agglutinin I, RCA I (specific for Galβ1,4GlcNAc), *Lens culinaris* agglutinin, LCA (specific for α1,6 core fucose). Generally, lectin-binding to both BPH FN and PCa FN was more noticeable when reduced samples were analyzed, probably due to exposure of lectin-reactive moieties.

Thus, the main immunoreactive BPH FN band at 220 kDa was bound by RCA I, SNA, Con-A and weakly by LCA (Fig. 2B). These lectins reacted differently with the three immunoreactive bands of PCa FN, observed (Fig. 2C). Thus, SNA and RCA I bound to the 90 kDa and 110 kDa bands, which were also less strongly recognized by Con-A and LCA, respectively. As for 140 kDa immunoreactive band, it was stained only with RCA I.

Microheterogeneity of the oligosaccharide chains of BPH FN and PCa FN was analyzed by lectin-affinity

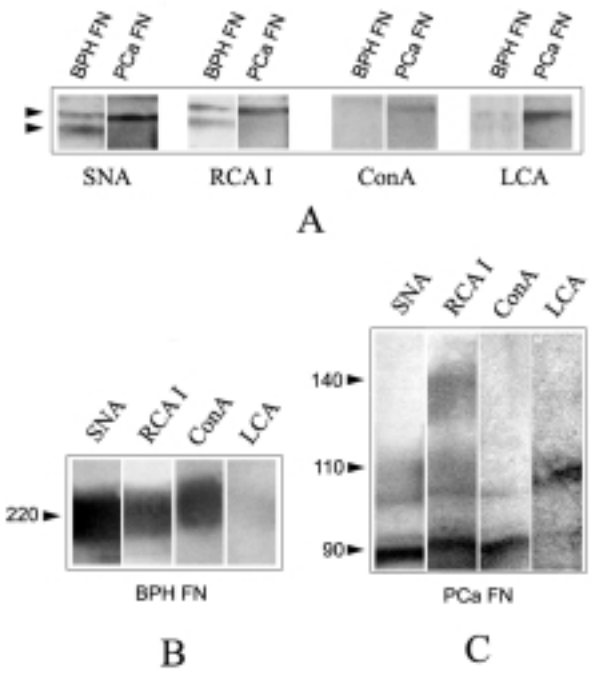

Fig. 2. Lectin-blot analysis of affinity-purified FN preparations. Fibronectin preparations, isolated from pools of BPH (pool I) and PCa (pool III) sera, by gelatin-affinity chromatography, were resolved on 6% separating and 3.75% stacking gel, blotted on membrane and probed with biotinylated lectins: SNA (*Sambucus nigra* agglutinin), RCA I (*Ricinus communis* agglutinin I), Con-A (lectin from *Canavalia ensiformis*) and LCA (*Lens culinaris* agglutinin). Bound lectins were visualized using a mixture of avidin and biotinylated-HRPO followed by addition of H_2O_2 and 3,3'-diaminobenzidine. Panel A: lectin blots of BPH FN and PCa FN, resolved under non-reducing conditions (see Fig. 1.A.1). Panel B: lectin blots of BPH FN, resolved under reducing conditions (see Fig. 1.A.2). Panel C: lectin blots of PCa FN, resolved under reducing conditions (see Fig. 1.A.2). Numbers indicate molecular mass (kDa).

chromatography using radiolabeled samples. The relative abundances of fractions eluted with sugar solution or low pH buffer, which separated high affinity bound glycoforms from weakly bound ones were, recorded (Fig. 3A, 3B). The results obtained indicated that the FN preparations comprised glycoforms differing in relative abundance and lectin-reactivities. The Con-A-reactive fraction of BPH FN was most abundant, followed by SNA- (46%) and RCA I- (32%) reactive fractions. The LCA-reactive fraction (20%) was the lowest (Fig. 3A). On the other hand, the relative amount of altogether immuno/lectin-reactive PCa FN was highest for RCA I- and SNA-, followed by Con-A- (72%) and LCA- bound fraction (59%) (Fig. 3B).

3.3. Adhesion of fibroblasts to BPH FN and PCa FN

COS-7 fibroblast adhesion to BPH FN and PCa FN-coated wells are shown in Fig. 4A. Cells adhered to both FN preparations in a concentration dependent manner (concentration range 0.2–26.5 μg/mL), but with a significantly lower efficiency to PCa FN coated wells (Fig. 4A).

Similar results were observed when pools of BPH and PCa sera were used as the FN source instead of affinity-purified preparations, suggesting that the effect observed was not due to molecular modification and degradation during experimental procedures. Thus, monoclonal anti-FN Ab were adsorbed to cell-culture plates and allowed to react with pools of BPH- or PCa-sera, after which COS-7 fibroblasts were added and incubated at 37°C for one hour to attach to Ab-bound FN (Fig. 4B). The results obtained indicated adhesion efficiency to BPH FN increased at concentrations between 4 μg/mL and 20 μg/mL, after which saturation was reached.

4. Discussion

The data we obtained for BPH FN and PCa FN pointed to differences in their protein and oligosaccharide

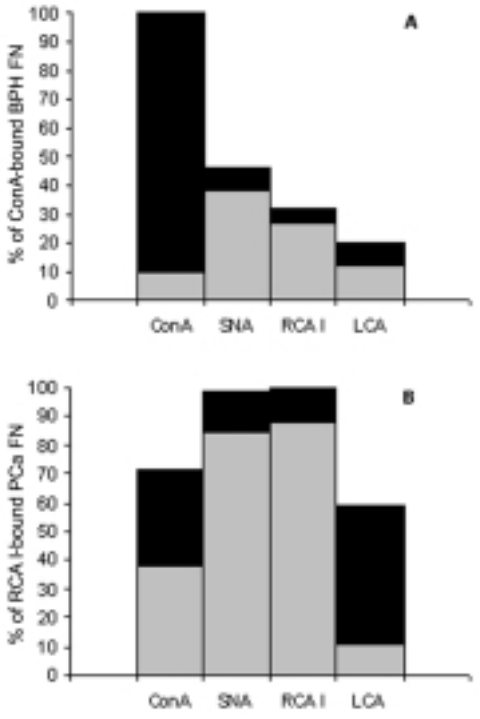

Fig. 3. Microheterogeneity of BPH FN and PCa FN: Fibronectin preparations, isolated from pools of BPH (pool I) and PCa (pool III) sera, by gelatin-affinity chromatography, were iodinated and subjected to lectin-affinity chromatography to detect the presence of different FN glycoforms. The following lectins were used as ligands: SNA (*Sambucus nigra* agglutinin), RCA I (*Ricinus communis* agglutinin I), Con-A (lectin from *Canavalia ensiformis*) and LCA (*Lens culinaris* agglutinin). A common chromatographic scheme was applied to all columns. The unbound and the retarded fractions were eluted with binding buffers. The bound fractions were specifically eluted by addition of competitive sugars or low pH buffers as indicated in Materials and methods. The percentage of sugar-elutable (grey) and low pH buffer-elutable (dark) fractions was calculated relative to the total bound radioactivity to the most reactive lectin. A) BPH FN; y-axis: % of BPH FN bound to ConA as the lectin most reactive with BPH FN; B) PCa FN; y-axis: % of PCa bound to RCA I as the lectin most reactive with PCa FN.

composition seen as distinct patterns of molecular mass forms and lectin-reactive forms.

BPH FN showed the pattern characteristic of normal plasma FN, which differed from PCa FN mainly in respect to the presence of FN fragments. Three immunoreactive fragments having molecular masses of 140, 110 and 90 kD were dominant, the last one detected specifically in PCa. Generally, FN fragments are reported to be characteristic of various pathological conditions. They can be generated through metalloproteinase or kallikrein activity and released from tissue into the circulation [20,21,37,38]. BPH and PCa are accompanied with elevated serum concentrations of kallikrein-3, but no correlation with FN concentration in cancer has been found [28]. However, the available experimental data are different, indicating both decrease and increase of FN level in cancer [26–28,38, 39].

FN is a glycoprotein containing 5% carbohydrates linked as both N- and O-glycans [14]. Depending on the source, FN exhibits differences in degree of sialylation and the type of linkage of sialic acid, as well as in the absence or presence of fucose [40–46]. The N-glycans of plasma FN are complex-type biannte-nary oligosaccharides, largely sialylated with predominance of the NeuAc alpha 2,6Gal linkage, whereas fetal/oncofetal FN, which is upregulated during development/transformation, is core fucosylated and generally not or poorly sialylated, with predominance of the NeuAc alpha 2,3Gal linkage. In addition, neoplastic transformation is generally accompanied by greater branching and increased carbohydrate content of FN, but the available data are mainly related to cellular FN forms, whereas data on plasma FN in cancer are scarce [47–51].

The analysis of the carbohydrate composition of BPH FN was in general agreement with the results reported for normal plasma FN regarding sialylation and fucosylation. In comparison to BPH FN, the ratio of altogether lectin-reactive PCa FN was different and manifested as a decrease of Con-A- and an increase of LCA-reactive forms. Since, PCa FN comprised different fragments, this should be born in mind when the results of glycosylation analysis are considered. PCa FN fragments have molecular masses corresponded to the cell-binding and gelatin-binding domain-containing fragments. It is known that the gelatin-binding domain on the FN molecule is mostly glycosylated, but the cell-binding domain also contains a significant amount of carbohydrates [14].

FN exhibits various biological activities through its different domains [12,14]. It is involved in cellular migration, adhesion, morphology and spreading, cytoskeletal organization, oncogenic transformation, phagocytosis, hemostasis/thrombosis embryonic differentiation etc [12,52]. These activities are mediated through its interactions with different molecules such as gelatin, heparin, integrins, actin, DNA, fibrin, hyaluronic acid, proteoglycans/gangliosides, thrombospondin, complement factor C1q [12,52]. Some of these activities might be influenced by glycosylation [12,14]. Thus, it was shown that inhibition of FN glycosylation increases its gelatin-binding and cell-

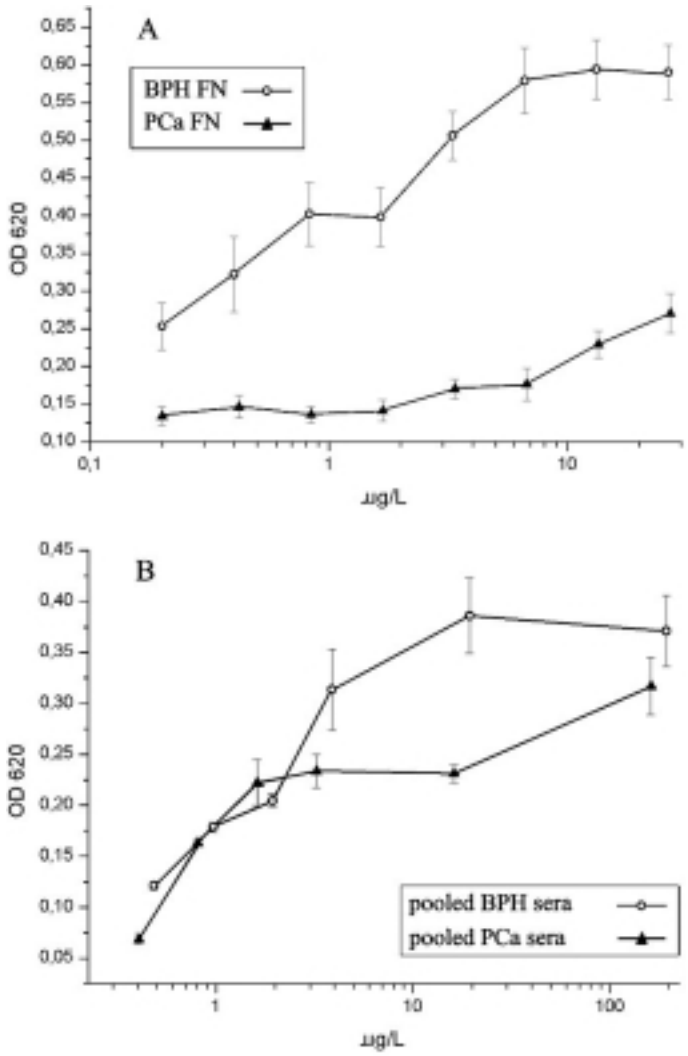

Fig. 4. COS-7 fibroblast adhesion to FN. A) Affinity purified FNs, isolated from pools of BPH (pool I) and PCa (pool III) sera, by gelatin-affinity chromatography, were adsorbed to cell-culture plates in the concentration range: 0.2–26.5 μg/mL. COS-7 fibroblasts were added (5×10^4 cells/well) and incubated for 1 hour at 37°C. After plating, cells were fixed, stained with Coomassie brilliant blue and then lysed with SDS after which the OD at 620 nm was measured. All samples were tested in duplicate, and the results are presented as the mean value with error bars representing the standard error.
B) Monoclonal anti-FN antibody, clone P1H11 were adsorbed to cell-culture plates (0.1 μg/well), and allowed to react with pools of BPH- or PCa -sera (FN concentration range: 0.4–162.0 μg/mL) for 1 hour at 37°C. COS-7 fibroblasts were added (5×10^4 cells/well) and incubated at 37°C for 1 hour to attach to antibody-bound FN. After plating, cells were fixed, stained with Coomassie brilliant blue and then lysed with SDS after which OD at 620 nm was measured. All samples were tested in duplicate, and the results are presented as the mean value with error bars representing the standard error.

spreading potency, but not heparin-binding, and that the presence of N-linked polylactosamine chains decreased the affinity for gelatin [53].

Our results showed that BPH FN and PCa FN differed in potency in the fibroblast adhesion assay, both as isolated preparations or as serum samples. Recent data indicated that the major fraction of FN present in ECM of tissue is plasma derived, suggesting that it has a general and direct role in cellular processes and maintenance of tissue integrity [54,55]. Concerning the molecular aspects of prostate cancer development, plasma FN is of interest due to its potency to stimulate invasion and metastatic spread and to protect prostate cancer cells from tumor necrosis factor [29,30,56–58]. Thus, the metastatic PCa cell, unlike the normal cell, can be stimulated by intact or distinct fibronectin frag-

ments to invade surrounding tissue.

The results obtained here showing some distinct structural characteristics of plasma FN in PCa compared to BPH could be important for modulation of its ligand and recognitive properties in terms of gaining or losing functions or as specific markers of its origin. This favors further work to explore these characteristics from both basic and clinical aspects.

Acknowledgement

The authors thank dr Snezana Kojic from IMGGE, for providing COS-7 cell line. This work was supported by the Ministry for Science of the Republic of Serbia, project code 143048.

References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu and M.J. Thun, Cancer statistics, 2007, *CA Cancer J Clin* **57**(**1**) (2007), 43–66.
[2] R.J. Black, F. Bray, J. Ferlay and D.M. Parkin, Cancer incidence and mortality in the European Union: cancer registry data and estimates of national incidence for 1990, *Eur J Cancer* **33** (1997), 1075–1107.
[3] C.J. Girman, Population-based studies of the epidemiology of benign prostatic hyperplasia, *Br J Urol* **82** (Supl 1) (1998), 34–43.
[4] G.W. Chodak, R.A. Thisted, G.S. Gerber, J.E. Johansson, J. Adolfsson, G.W. Jones, G.D. Chisholm, B. Moskovitz, P.M. Livne and J. Warner, Results of conservative management of clinically localized prostate cancer, *N Engl J Med* **330**(4) (1994), 242–248.
[5] M.J. Barry, F.J. Jr. Fowler, L. Bin, J.C. 3rd Pitts, C.J. Harris and A.G. Jr. Mulley, The natural history of patients with benign prostatic hyperplasia as diagnosed by North American urologists, *J Urol* **157**(1) (1997), 10–14.
[6] G.W. Cannon and R.H. Getzenberg, Biomarkers for benign prostatic hyperplasia progression, *Current Prostate Reports* **5**(3) (2007), 121–125.
[7] J.V Tricoli, M. Schoenfeldt and B.A, Detection of prostate cancer and predicting progression: current and future diagnostic markers, *Clin Cancer Res* **10**(12Pt1) (2004), 3943–3953.
[8] H.G. van der Poel, Molecular markers in the diagnosis of prostate cancer, *Crit Rev Oncol Hematol* **61**(2) (2007), 104–139.
[9] M.M. Janković and M.M. Kosanović, Glycosylation of urinary prostate-specific antigen in benign hyperplasia and cancer: assessment by lectin-binding patterns, *Clin Biochem* **38**(1) (2005), 58–65.
[10] S. Golubovic and M. Jankovic, Glycobiochemical characterization of salivary carcinoembryonic antigen, *J BUON* **12**(2) (2007), 269–275.
[11] D.F. Mosher, Fibronectin, *Prog Hemost Thromb* **5** (1980), 111–151.
[12] R.O. Hynes and K.M. Yamada, Fibronectins: multifunctional modular glycoproteins, *J Cell Biol* **95**(2 Pt 1) (1982), 369–377.
[13] C. Ffrench-Constant, Alternative splicing of fibronectin-many different proteins but few different functions, *Exp Cell Res* **221**(2) (1995), 261–271.
[14] R. Pankov and K.M. Yamada, Fibronectin at a glance, *J Cell Sci* **115**(Pt 20) (2002), 3861–3863.
[15] U. Koçak, U. Ezer and S. Vidinlisan, Serum fibronectin in neonatal sepsis: is it valuable in early diagnosis and outcome prediction? *Acta Paediatr Jpn* **39**(4) (1997), 428–432.
[16] M.E. Lancer and G.E. Brown, Induction of hepatocytes synthesis of fibronectin by a non-interleukin 1 monokine, *Journal of Trauma* **28**(8) (1988), 1220–1225.
[17] F. Boccardo, D. Guarneri, S. Zanardi, P. Castellani, L. Borsi and L. Zardi, Fibronectin concentration in the plasma of patients with malignant and benign breast disease, *Cancer Lett* **33**(3) (1986), 317–323.
[18] S. Jung, G. Jung, A. Tranzer and R. Dörr, Blood fibronectin changes in various neoplasms, *Presse Med* **15**(5) (1986), 197–198, 203–204.
[19] C. Haglund, S. Ylätupa, P. Mertaniemi and P. Partanen, Cellular fibronectin concentration in the plasma of patients with malignant and benign diseases: a comparison with CA 19-9 and CEA, *Br J Cancer* **76**(6) (1997), 777–783.
[20] J. Labat-Robert, Fibronectin in malignancy, *Semin Cancer Biol* **12**(3) (2002), 187–195.
[21] F. Grinnell and M. Zhu, Identification of neutrophil elastase as the proteinase in burn wound fluid responsible for degradation of fibronectin, *J Invest Dermatol* **103**(2) (1994), 155–161.
[22] M.L. Barilla and S.E. Carsons, Fibronectin fragments and their role in inflammatory arthritis, *Semin Arthritis Rheum* **29**(4) (2000), 252–265.
[23] J.M. Guo, X.Y. Zhang, H.L. Chen, G.M. Wang and Y.K. Zhang, Structural alterations of sugar chains in urine fibronectin from bladder cancer patients and its enzymatic mechanism, *J Cancer Res Clin Oncol* **127**(8) (2001), 512–519.
[24] A. Hegele, A. Heidenreich, J. Kropf, R. von Knobloch, Z. Varga, R. Hofmann and P. Olbert, Plasma levels of cellular fibronectin in patients with localized and metastatic renal cell carcinoma, *Tumor Biol* **25**(3) (2004), 111–116.
[25] U.M. Warawdekar, S.M. Zingde, K.S. Iyer, P. Jagannath, A.R. Mehta and N.G. Mehta, Elevated levels and fragmented nature of cellular fibronectin in the plasma of gastrointestinal and head and neck cancer patients, *Clin Chim Acta* **372**(1–2) (2006), 83–93.
[26] H. Sönmez, S. Süer, I. Karaarslan, H. Baloğlu and E. Kökoğlu, Tissue fibronectin levels of human prostatic cancer, as a tumor marker, *Cancer Biochem Biophys* **15**(2) (1995), 107–110.
[27] M. Albrecht, H. Renneberg, G. Wennemuth, O. Möschler, M. Janssen, G. Aumüller and L. Konrad, Fibronectin in human prostatic cells in vivo and in vitro: expression, distribution, and pathological significance, *Histochem Cell Biol* **112**(1) (1999), 51–61.
[28] S. Chakrabarty and H.A. Fritsche, Fibronectin and prostate cancer, *Journal of Clinical Ligand Assay* **25** (2002), 74–79.
[29] M. Morgan, S. Saba and W. Gower, Fibronectin influences cellular proliferation and apoptosis similarly in LNCaP and PC-3 prostate cancer cell lines, *Urol Oncol* **5**(4) (2000), 155–159.
[30] D.L. Livant, R.K. Brabec, K.J. Pienta, D.L. Allen, K. Kurachi, S. Markwart and A. Upadhyaya, Anti-invasive, antitumorigenic, and antimetastatic activities of the PHSCN sequence in prostate carcinoma, *Cancer Res* **60**(2) (2000), 309–320.
[31] E. Ruoslahti, E.G. Hayman, M. Pierschbacher and E. Engvall, Fibronectin: purification, immunochemical properties, and

biological activities, *Methods Enzymol* **82** (Pt A) (1982), 803–831.
[32] U. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**(1970), 680–685.
[33] P.C. Greenwood, M.W. Hunter and S. Glover, The preparation of ^{131}I labelled growth hormone of high specific radioactivity, *Biochem J* **89** (1963), 114–120.
[34] Y. Gluzman, SV40-transformed simian cells support the replication of early SV40 mutants, *Cell* **23**(1) (1981), 175–182.
[35] http://www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=CRL-1651.
[36] M. Hyytiäinen and J. Keski-Oja, Latent TGF-beta binding protein LTBP-2 decreases fibroblast adhesion to fibronectin, *J Cell Biol* **163**(6) (2003), 1363–1374.
[37] I. Katnik-Prastowska, M. Przybysz, A. Chełmońska-Soyta, Fibronectin fragments in human seminal plasma, *Acta Biochim Pol* **52**(2) (2005), 557–560.
[38] M.M. Webber, A. Waghray and D. Bello, Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion, *Clin Cancer Res* **1**(10) (1995), 1089–1094.
[39] http://www.copewithcytokines.de/cope.cgi?key=Fibronectin.
[40] Y. Yamaguchi, M. Isemura, M. Kosakai, A. Sato, M. Suzuki, M. Kan and Z. Yosizawa, Characterization of fibronectin from fetal human plasma in comparison with adult plasma fibronectin, *Biochim Biophys Acta* **790**(1) (1984), 53–60.
[41] T. Krusius, M. Fukuda, A. Dell and E. Ruoslahti, Structure of the carbohydrate units of human amniotic fluid fibronectin, *J Biol Chem* **260**(7) (1985), 4110–4116.
[42] S. Carsons, B.B. Lavietes, A. Slomiany, H.S. Diamond and E. Berkowitz, Carbohydrate heterogeneity of fibronectins. Synovial fluid fibronectin resembles the form secreted by cultured synoviocytes but differs from the plasma form, *J Clin Invest* **80**(5) (1987), 1342–1349.
[43] E. Köttgen, B. Hell, C. Müller, F. Kainer and R. Tauber, Developmental changes in the glycosylation and binding properties of human fibronectins. Characterization of the glycan structures and ligand binding of human fibronectins from adult plasma, cord blood and amniotic fluid, *Biol Chem Hoppe Seyler* **370**(12) (1989), 1285–1294.
[44] M. Takamoto, T. Endo, M. Isemura, N. Kochibe and A. Kobata, Structures of asparagine-linked oligosaccharides of human placental fibronectin, *J Biochem* (*Tokyo*) **105**(5) (1989), 742–750.
[45] M. Takamoto, T. Endo, M. Isemura, Y. Yamaguchi, K. Okamura, N. Kochibe and A. Kobata, Detection of bisected biantennary form in the asparagine-linked oligosaccharides of fibronectin isolated from human term amniotic fluid, *J Biochem* (*Tokyo*) **106**(2) (1989), 228–235.
[46] M . Tajiri, S. Yoshida and Y. Wada, Differential analysis of site-specific glycans on plasma and cellular fibronectins: application of a hydrophilic affinity method for glycopeptide enrichment, *Glycobiology* **15**(12) (2005), 1332–1340.
[47] K. Murayama, E.J. Nichols, S.B. Levery, W.G. Carter and S. Hakomori, The carbohydrate structure of human fibronectins: A comparison between normal embryonic lung fibroblasts WI-38 and the SV40 virus transformed cell line VA13, *Glycoconjugate Journal* **1**(2) (1984).
[48] H. Matsuura and S. Hakomori, The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma, *Proc Natl Acad Sci USA* **82**(19) (1985), 6517–6521.
[49] E.J. Nichols, B.A. Fenderson, W.G. Carter and S. Hakomori, Domain-specific distribution of carbohydrates in human fibronectins and the transformation-dependent translocation of branched type 2 chain defined by monoclonal antibody C6, *J Biol Chem* **261**(24) (1986), 11295–11301.
[50] H. Matsuura, T. Greene and S. Hakomori, An alpha-N-acetylgalactosaminylation at the threonine residue of a defined peptide sequence creates the oncofetal peptide epitope in human fibronectin, *J Biol Chem* **264**(18) (1989), 10472–10476.
[51] U. Mandel, Hamilton, M. Therkildsen, J. Reibel, B. Sweeney, H. Matsuura, S. Hakomori, E. Dabelsteen and H. Clausen, Cancer-associated changes in glycosylation of fibronectin. Immunohistological localization of oncofetal fibronectin defined by monoclonal antibodies, *APMIS* **100**(9) (1992), 817–826.
[52] http://home.comcast.net/˜kennethingham/newsite/intro.
[53] G.E. Jones, R.G. Arumugham and M.L Tanzer, Fibronectin glycosylation modulates fibroblast adhesion and spreading, *J Cell Biol* **103**(5) (1986), 1663–1670.
[54] A.J. Millis, M. Hoyle, D.M. Mann and M.J. Brennan, Incorporation of cellular and plasma fibronectins into smooth muscle cell extracellular matrix *in vitro*, *Proc Natl Acad Sci USA* **82**(9) (1985), 2746–2750.
[55] F.A. Moretti, A.K. Chauhan, A. Iaconcig, F. Porro, F.E. Baralle and A.F. Muro, A major fraction of fibronectin present in the extracellular matrix of tissues is plasma-derived, *J Biol Chem* **282**(38) (2007), 28057–28062.
[56] M. Yi and E. Ruoslahti, A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis, *Proc Natl Acad Sci USA* **98**(2) (2001), 620–624.
[57] M. Fornaro, J. Plescia, S. Chheang, G. Tallini, Y.M. Zhu, M, King, D.C. Altieri and L.R. Languino, Fibronectin protects prostate cancer cells from tumor necrosis factor-alpha-induced apoptosis via the AKT/survivin pathway, *J Biol Chem* **278**(50) (2003), 50402–50411.
[58] K.L. van Golen, L. Bao, G.J. Brewer, K.J. Pienta, J.M. Kamradt, D.L. Livant and S.D. Merajver, Suppression of tumor recurrence and metastasis by a combination of the PHSCN sequence and the antiangiogenic compound tetrathiomolybdate in prostate carcinoma, *Neoplasia* **4**(5) (2002), 373–379.