Abstract. Prostate carcinomas are one of the most common malignancies in western societies. The pathogenesis of this tumor is still poorly understood. These tumors present with two characteristic features: epithelial-mesenchymal interactions, which play a pivotal role for tumor development and most of clinically manifest cancers arise in prostate proper compared to a minority of tumors which develop in the transitional zone. Deciphering the epithelial-mesenchymal cross talk and identification of molecular peculiarities of the sub-populations of cells in different zones can therefore help understanding carcinogenesis and development of new, non-invasive tools for the diagnosis and prognosis of prostate carcinomas which has remained a challenge until today. A ProteinChip® array technology (SELDI = surface enhanced laser desorption ionization) has been developed recently by Ciphergen Biosystems enabling analysis and profiling of complex protein mixtures from a few cells. This study describes the analysis of approximately 500-1000 freshly obtained prostate cells by SELDI-TOF-MS (surface enhanced laser desorption ionization time-of-flight mass spectrometry). Pure cell populations of stroma, epithelium and tumor cells were selected by laser assisted microdissection. Multiple specific protein patterns were reproducibly detected in the range from 1.5 to 30 kDa in 28 sub-populations of 4 tumorous prostates and 1 control. A specific 4.3 kDa peak was increased in the prostate tumor stroma compared to normal prostate proper and transitional zone stroma and increased in prostate tumor glands compared to normal prostate proper and transitional zone glands. Coupling laser assisted microdissection with SELDI provides tremendous opportunities to identify cell and tumor specific proteins to understand molecular events underlying prostate carcinoma development. It underlines the vast potential of this technology to better understand pathogenesis and identify potential candidates for new specific biomarkers in general which could help to screen for and distinguish disease entities, i.e. between clinically significant and insignificant carcinomas of the prostate.

Introduction

Prostate cancer has become one of the most commonly diagnosed cancers in the United States and one of the leading causes of cancer death in North America and Western Europe (1). The tumor has two characteristic features: it develops within a complex epithelial-mesenchymal tissue with well coordinated intercellular cross talks (2) and up to 70% of prostate carcinomas arise in the posterolateral (peripheral) zone of the prostate, the prostate proper (3), whereas only about 25% develop in the transitional zone. Most of these tumors are incidental carcinomas. Even though there are no obvious histomorphological differences between those zones, important molecular peculiarities from one zone to the other must be postulated which favor development of carcinomas in the prostate proper.

Early diagnosis of prostate carcinomas is of great importance for management and outcome of disease and cost efficient and specific cancer biomarkers are important tools for screening and monitoring especially with a shrinking health care budget. Today, the most reliable ways of diagnosis of prostate carcinomas are palpation, ultrasound guided biopsy with subsequent histology and serum marker analysis. Prostate specific antigen (PSA) is considered to be the best marker presently available. However it does not always correctly distinguish between benign and malignant prostate.
diseases. The fact that significant prostate cancer can be missed by PSA determination (4) may be based on the molecular heterogeneity of these tumors and an underlying differential protein expression pattern. Therefore identification and application of a panel of novel biomarkers has great potential for improving tumor management. Novel biomarkers are often identified by studying molecular pathogenesis. Today increasing emphasis is laid upon the analysis of cellular proteomics in addition to transcriptional studies with cDNA arrays, which can accelerate identification of new diagnostic markers (5). The drawback of cDNA arrays is, that they detect intermediate molecules, not the effector targets, and for this reason all detected differences have to be confirmed by some means on the level of protein expression. Since the correlation between RNA and protein expression is about 0.48, there are many false positives among the identified possible targets.

A classical way of discovering new diagnostic tools and markers on the protein level is 2D polyacrylamide gel electrophoresis (2D PAGE) (6,7). However, although 2D PAGE is able to resolve thousands of proteins, it is labor intensive, lacks reproducibility and requires large amounts of starting material. In contrast, better understanding of prostate carcinoma development in particular requires separation of stromal and epithelial cells. Therefore, sub-populations of cells have to be selected by some technology for further experiments like tissue proteomics studies. Laser assisted micromanipulation is one of those tools enabling identification and isolation of specific cells under permanent visual control (8,9).

The development of SELDI-TOF-MS has overcome many limitations of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and 2D PAGE (10). This technology reverses the conventional MALDI sample preparation by using a ProteinChip array of addressable protein binding sites on a solid substrate. Captured individual proteins from complex mixtures are subsequently resolved by mass spectrometry. This fast, novel technology combines several advantages over 2D Gels, most importantly, it requires far less starting material, and has a higher reproducibility. In the low mass range proteins can be analyzed in the femtomole range. Further SELDI-TOF-MS is directly applicable for clinical assay development. Its efficacy to identify potential markers for prostate and bladder carcinoma has been shown recently (11-14).

This report describes our initial studies using laser assisted microdissection in conjunction with SELDI and a novel elaborated analysis software to detect potential new diagnostic markers for prostate carcinomas by trying to understand further aspects of carcinogenesis.

Materials and methods

Tissue procurement. Radical prostatectomy specimens were obtained from 4 patients with carcinoma and 1 control (obtained with a cystectomy) after informed consent. All identifiers were removed from samples and they were analyzed anonymously. None of the patients had been treated with radio-, chemotherapy or ablative hormonal therapy. Patients age, Gleason scores and tumor stages are shown in Table I. A staff pathologist from the Institute of Pathology, University of Bonn was present in the operation room when prostates were surgically removed by urologists from the Urology Department of the University of Bonn. Immediately, prostates were sliced. Samples from the prostate proper and transitional zone were snap frozen in -180°C liquid nitrogen. Areas macroscopically suspicious of tumor were also sampled. This procedure assured that not more than 5-10 min passed from final surgical removal of the organ to final freezing of the samples. Further preparation of the organ by a surgical pathologist and their histological evaluation guaranteed correct grading, TNM classification and application of all diagnostic standards.

Laser microdissection. Populations of epithelial and mesenchymal cells from different zones and tumor cells, altogether 28 sub-populations, were isolated from frozen tissue sections using a PALM System (PALM AG, Bernried, Germany) by a pathologist (Fig. 1). Based on careful review of histologic sections the cell samples contained more than 95% of desired cells.

SELDI analyses of cell lysates. A total of 1500 microdissected cells were lysed in 15 µl of 50 mM HEPES with 1% Triton X-100 (pH 7.4). The lysates were homogenized by vigorous vortexing, vigorous resuspending and subsequently centrifuged for 5 min at maximum speed with a bench top centrifuge. The spots of a SAX-2 (strong anionic exchange surface using quaternary ammonium groups) ProteinChip array were pre-incubated with PBS (pH 7.5). PBS was replaced after 5 min by 5 µl of the resulting supernatant after centrifugation and then incubated for 30 min at room temperature in a humidity chamber. After incubation the lysate was withdrawn and the spots washed 2 times with PBS + 0.05% Triton, then washed twice with aqua bident. Mass analysis was performed with the Protein Biology System II using 2 applications of saturated sinapinic acid (SPA) dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid (TFA). Twice 0.7 µl of TFA was applied to each spot after drying. ProteinChip arrays were analyzed in a ProteinChip reader according to an automated data collection protocol. The instrument was operated with a source and detector voltage of 20 and 1.8 kV respectively. Laser intensity was set to 250, detector sensitivity to 10 with source and detector voltage of 20 and 1.8 kV respectively. Laser microdissection. Populations of epithelial and mesenchymal cells from different zones and tumor cells, altogether 28 sub-populations, were isolated from frozen tissue sections using a PALM System (PALM AG, Bernried, Germany) by a pathologist (Fig. 1). Based on careful review of histologic sections the cell samples contained more than 95% of desired cells.

Table I. Summary of patient age, tumor stage and Gleason scores.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Tumor stage</th>
<th>Gleason score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>58</td>
<td>pT2b pN0</td>
<td>3+3=6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>59</td>
<td>pT3a pN0</td>
<td>3+3=6</td>
</tr>
<tr>
<td>Patient 3</td>
<td>64</td>
<td>pT2a pN0</td>
<td>2+3=5</td>
</tr>
<tr>
<td>Patient 4</td>
<td>62</td>
<td>pT2b pN1</td>
<td>4+5=9</td>
</tr>
<tr>
<td>Patient control</td>
<td>73</td>
<td>pT0</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The Table I. shows the summary of patient age, tumor stage, Gleason scores and their correlation.
fired, 65 of them collected starting at position 21 and ending at position 81 of the spots. Data interpretation was augmented using the ProteinChip software version 2.1b. In detail the spectra obtained under similar experimental conditions from all 28 sub-populations on SAX-2 chips were imported in one experiment file and the peaks automatically labeled (automatic peak detection) with highest detection sensitivity. The samples were grouped related to the different sub-populations and subsequently combination maps were generated by the software combining all spectra of 1 sub-population into 1 combination map using the following parameters: peak closeness 0.5% of mass, compare peak height as average of intensity. The resulting combination maps were then compared groupwise to produce comparison maps. The results of the combination and comparison maps are shown in Fig. 3. We were able to detect a number of differentially regulated proteins in the mass range between 1.5 and 30 kDa. The most prominent up-regulated peak in the low molecular weight mass range was a single peak with a mass of 4299 Da (mass error ± 1000 ppm due to calibration reasons). The relative intensity of the peak height in all spectra is shown again in Table II. The average relative intensity of the 4299 Da peak in the combined tumor gland samples was 24.37, in the normal prostate proper samples 7.26, and in the normal transitional zone gland samples 9.99. In the combined tumor stroma samples the average relative intensity was 41.51, in the prostate proper stroma 7.06 and in the transitional zone stroma 5.9 (Table IIb).

Table IIa shows the absolute differences of the averaged peak intensities when compared groupwise. The 4300 Da peak is increased in tumor glands vs. normal prostate proper glands (17.11), tumor glands vs. normal transitional zone glands (14.38), decreased in normal prostate proper glands vs. normal transitional zone glands (-2.73) and increased again in tumor stroma vs. normal prostate proper stroma (34.45), tumor stroma vs. normal transitional zone stroma (35.61) and normal prostate proper stroma vs. normal transitional zone stroma (1.16).

We measured a 3.4-fold up-regulation of the 4299 Da peak in tumor glands vs. normal prostate proper glands, and 2.43-fold up-regulation in tumor glands vs. normal transitional zone glands compared to almost no up-regulation between normal prostate proper glands/normal transitional zone glands.

Results

To determine sensitivity limitations of SELDI-TOF-MS we performed a pilot study and tested a panel of extracts obtained from less than 500 cells. These extracts contained enough protein lysate to generate sufficient reliable protein profiles as we observed spectra similar to those obtained from more cells. Therefore we concluded, that the PALM instrument was in general applicable for our project. Further data were generated using the equivalent amount of protein of approximately 500 cells for binding on the spots of the SAX-2 ProteinChips (per extract).

Processing on a strong anionic exchange surface (SAX-2) resolved up to 100 peaks in the mass range between 1 and 30 kDa (data not shown). Fig. 2 demonstrates representative protein mass spectra showing protein profiles from 1.5 to 5 kDa. For data analysis software features of the ProteinChip software version 2.1b were used. The spectra obtained under similar experimental conditions from all 28 sub-populations on SAX-2 chips were imported in one experiment file and the peaks automatically labeled (automatic peak detection) with highest detection sensitivity. The results for the 1500 to 5000 Da mass ranges are shown in Fig. 2. The samples were grouped related to the different sub-populations and subsequently combination maps were generated by the software combining all spectra of 1 sub-population into 1 combination map. The resulting combination maps were then compared groupwise to produce comparison maps. The results of the combination and comparison maps are shown in Fig. 3. We were able to detect a number of differentially regulated proteins in the mass range between 1.5 and 30 kDa. The most prominent up-regulated peak in the low molecular weight mass range was a single peak with a mass of 4299 Da (mass error ± 1000 ppm due to calibration reasons). The relative intensity of the peak height in all spectra is shown again in Table II. The average relative intensity of the 4299 Da peak in the combined tumor gland samples was 24.37, in the normal prostate proper samples 7.26, and in the normal transitional zone gland samples 9.99. In the combined tumor stroma samples the average relative intensity was 41.51, in the prostate proper stroma 7.06 and in the transitional zone stroma 5.9 (Table IIb).

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Figure 2. Representative protein mass spectra showing protein profiles from 1.5 to 5 kDa.
Figure 3. Combination and comparison maps of all 28 sub-populations as indicated (mass range 3500-5000 Da). The resulting combination maps were compared groupwise to produce comparison maps using the following parameters: peak closeness, 1%; peak height, intensity; peaks only in spectrum A, in blue color in positive direction; peaks only in spectrum B, in green color showing in negative direction; and peaks in spectrum A and B, in red color showing in both directions depending on the difference in peak height in the spectra A and B respectively. Spectra 1-3 show the comparison maps tumor glands/normal prostate proper glands (spectrum 1), tumor glands/normal transitional zone glands (spectrum 2) and normal prostate proper glands/normal transitional zone glands (spectrum 3). The respective combination maps are shown in spectra 4-6 (combination tumor glands, spectrum 4; combination normal prostate proper glands, spectrum 5; combination normal transitional zone glands, spectrum 6). Spectra 10-12 show the comparison maps tumor stroma/normal prostate proper stroma (spectrum 10), tumor stroma/normal transitional zone stroma (spectrum 11) and normal prostate proper stroma/normal transitional zone stroma (spectrum 12). The combination maps are shown in spectrum 7 (combination tumor stroma), spectrum 8 (combination normal prostate proper stroma) and spectrum 9 (combination normal transitional zone stroma).
Table II. a, Average intensities of the 4299 Da peak in the compared sample groups.

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Average Intensity</th>
</tr>
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<tbody>
<tr>
<td>Tumor glands-normal prostate proper g</td>
<td>17.11</td>
</tr>
<tr>
<td>Tumor glands-normal transitional g</td>
<td>14.38</td>
</tr>
<tr>
<td>Normal prostate proper glands-normal</td>
<td>-2.73</td>
</tr>
<tr>
<td>Transitional glands</td>
<td></td>
</tr>
<tr>
<td>Tumor stroma-normal prostate stroma</td>
<td>34.45</td>
</tr>
<tr>
<td>Tumor stroma-normal transitional stroma</td>
<td>35.61</td>
</tr>
<tr>
<td>Normal prostate proper stroma-normal</td>
<td>1.16</td>
</tr>
</tbody>
</table>

b, The relative intensity of the 4299 Da peak height in different cell population.

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Average Intensity</th>
<th>% Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor glands</td>
<td>24.37</td>
<td>86.15</td>
</tr>
<tr>
<td>Normal transitional glands</td>
<td>9.99</td>
<td>117.34</td>
</tr>
<tr>
<td>Normal prostate proper glands</td>
<td>7.26</td>
<td>79.9</td>
</tr>
<tr>
<td>Normal prostate proper stroma</td>
<td>7.06</td>
<td>49.9</td>
</tr>
<tr>
<td>Tumor stroma</td>
<td>41.51</td>
<td>28.79</td>
</tr>
<tr>
<td>Normal transitional stroma</td>
<td>5.9</td>
<td>66.92</td>
</tr>
</tbody>
</table>

(0.72 times). A similar observation was made in the tumor stroma. Here we measured a 5.88 up-regulation vs. normal prostate proper stroma and a 7.04 up-regulation compared to normal transitional zone stroma.

Discussion

The challenging search for molecular differences between specific sub-populations of cells within the prostate and prostate carcinoma cells will not only help to understand carcinogenesis but identify biomarkers that could support tumor diagnosis and screening. Ultimately it will improve management of this very frequently occurring disease. Studies of this kind have been hampered, among other reasons, by the need of large amounts of proteins for analysis by traditional 2D Gel electrophoresis or cDNA arrays. Especially from tissue it used to be very tedious to procure larger amounts of specific cell populations. However, the development of laser supported microdissection overcome some of the problems to generate homogeneous cell populations (8,15). The development of the SELDI technology finally offers researchers a very sensitive tool to analyze complex protein mixtures from just a few cells.

Using the commercially available ProteinChip software version 2.1b for the first time for microdissected tissue we have identified several differences in protein mass spectra of the different zones of the human prostate suggesting that there are indeed fundamental molecular differences among those zones, one of them a protein peak with a molecular weight of 4299 Da. This one was clearly differentially expressed both in the tumorous epithelial and stroma compared to their normal counterparts. Since this protein interestingly seems to be expressed both in tumor cells and adjacent stroma but not in the normal counterparts suggests an important role for tumorigenesis. Based on our study we will now address the identification of the 4299 Da peak and of the other differentially expressed peaks. Identification could be done with a technology recently developed at Ciphergen Biosystems Inc. which was used also by another group to identify a potential biomarker discovered in seminal plasma. There a 5751 Da potential biomarker was enriched on a mixed mode surface and the doubly charged species (2876 Da) was used to identify this candidate by LDI Qq-TOF analysis to be the human seminal basic protein fragment of human semenogelin I (16). We also found potential candidates in the prostate proper and transitional zone which could be correlates of molecular peculiarities of these areas despite their lack of histomorphological differences.

If one of the differentially expressed proteins related to tumor is secreted one could try to develop a novel biomarker for serum diagnosis.

In general our data provide further evidence for findings also reported by Wright et al (12) and Paweletz and Petricoin (17). They have applied SELDI technology to detect up-regulated potential new biomarkers from cell lysates of pure populations of prostate cancer-associated cells (epithelial cells) procured by laser capture microdissection (LCM) (Arcturus Inc.) when compared with mass spectra of normal cell lysates. They detected novel proteins to be up-regulated in prostate cancer epithelial cells compared to normal cells beside already known prostate cancer-associated biomarkers (prostate specific antigen, prostate specific peptide, prostate acid phosphatase and prostate specific membrane antigen). One protein with a mass of 33436 Da appeared in 6 prostate adenocarcinoma LCM samples compared to matched normal cell lysates. They detected novel proteins to be up-regulated in prostate cancer epithelial cells compared to normal cells and transitional zone which could be correlates of molecular peculiarities of these areas despite their lack of histomorphological differences.

In summary, our study supports the applicability of this powerful new technology in conjunction with microdissection for protein profiling not only for prostate carcinoma but also for the different epithelial and stromal compartments of different zones of the prostate. It supported the findings, that not only LCM but also the PALM system can be used in conjunction with SELDI technology. This approach might generate important information allowing to better understand prostate cancer development. Also, we found the technology highly reproducibly and rapid. Usually an analysis was completed within a few hours. Most important within the experimental flow seems to us the procurement of absolutely fresh tissue. The presence of a staff surgical pathologist in the operation room underscores our conviction that despite procurement of fresh tissue diagnostic accuracy must not be impaired by using the patient material for research purposes.
The linkage of laser assisted microdissection and SELDI has given researchers the ability to resolve proteins from complex mixtures of less than 1000 cells on a high throughput basis. However, we feel that using less cells may even be biologically not significant. Also the software features of the peaks software 2.1b efficiently help to detect differences in relative peak height among all the spectra. This facilitates data analysis of a large number of spectra and offers tremendous opportunities to identify differences in protein expression associated with specific cell types.

Our study did not address absolute quantitative differences in protein expression. This is due to technical reasons, i.e. that detection of quantitative differences has not been standardized and is more challenging. But the development of a reliable method of protein quantification is an ongoing effort not only by Ciphergen Inc. (oral communication Dr Fung, Ciphergen Inc.) and will probably be available in the near future.

Future studies should possibly also consider socioeconomic implications of wide spread screening with new biomarkers, which will greatly increase overall health care costs (18). It is absolutely necessary for any health care provider to diagnose and stage not only malignancies but any disease in a cost effective manner. Therefore emphasis should not exclusively put on analysis of pathogenesis but also upon identification of markers in i.e. prostate carcinomas, capable of differentiation between clinically significant and insignificant carcinomas. Large randomized prospective screening trials in conjunction with the combination of microdissection and SELDI-TOF-MS could be supportive for identification of clinically important and cost effective novel markers.

Acknowledgements

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References