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·*Original Article*·

Identification of potential biomarkers of Peyronie's disease

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Abstract

Aim: To identify proteins that are differentially expressed in cells derived from normal and diseased tunica albuginea (TA) as related to Peyronie's disease (PD). **Methods:** Cells with characteristics of fibroblasts were isolated from two tissue sources. Those from the plaque of patients with PD were designated as PT cells, and those from the normally-appearing TA of the same patients were designated as NT cells. Messenger RNAs of these cells were analyzed by real-time polymerase chain reaction (RT-PCR) for the expression of monocyte chemoattractant protein 1 (MCP-1). Crude protein lysates were analyzed by surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) with IMAC30-Cu, CM10, and H50 chips. Each lysate was then separated into six fractions, which were further analyzed by SELDI-MS. **Results:** RT- PCR analysis showed that PT cells expressed higher levels of MCP-1 than their counterpart NT cells. SELDI-MS analysis showed that the crude protein lysates of all four cell strains produced similar and reproducible protein profiles on IMAC30-Cu and CM10 chips. Additional SELDI-MS analyses with the fractionated lysates detected three proteins of 11.6 kDa, 14.5 kDa, 22.6 kDa that were upregulated in PT cells and two proteins of 6.3 kDa and 46.9 kDa that were downregulated in PT cells. **Conclusion:** MCP-1, which is often involved in tissue fibrosis, was expressed at higher levels in PT than that in NT cells. Five potential biomarkers for PD were identified by SELDI-MS analysis. *(Asian J Androl 2005 Sep; 7: 237–243)*

Keywords: Peyronie's disease; MCP-1; tunica albuginea; penis; mass spectrometry; SELDI

1 Introduction

The tunica albuginea (TA) is a bi-layered structure that encloses the corpora cavernosa of the penis [1]. The TA's elasticity permits the increase in girth and length of the penis and its tensile strength provides the rigidity for the erect penis. However, this compliance is compromised by a condition called Peyronie's disease (PD) that afflicts 1 % –3 % of men and is characterized by a fibrotic lesion (plaque) in the TA, resulting in penile curvature during erection and, in severe cases, inability to maintain erection [2–4]. Possible causes of PD include vitamin E deficiency, the use of β-blockers, autoimmune response and genetic disorder. However, the most plau-

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sible cause is believed to be repetitive injuries to the penis during intercourse followed by hyperactive wound healing that results in excess extracellular matrix (ECM) production and disorganized ECM deposition in the TA, thereby forming scar-like tissues [5].

To investigate the pathogenesis of PD at the molecular level, we and other researchers [6–8] have adopted a cell culture model, in which cells with characteristics of fibroblasts are isolated from normal and diseased TA, and then they are cultivated, treated with agents, and/or analyzed for differentially expressed genes. In a previous study [8] we employed three types of cells: P-cells from the plaque, C-cells from the normal-appearing tissue near the plaque (C denotes the corners of the H-shaped incision in the venous grafting procedure [9, 10]), and N-cells from the TA of patients without PD. We found that MCP-1, a small protein of the C-C chemokine superfamily, was expressed at highest and lowest levels in P- and N-cells, respectively. We further demonstrated that the treatment of these three types of cells with TGF-β1 resulted in the upregulation of MCP-1.

In the present study, we established two new pairs of TA-derived cells and demonstrated that Peyronie's plaque-derived cells again expressed more MCP-1 than normal TA-derived cells. Then the protein profiles of these cells were compared using the surface-enhanced laser desorption/ionization (SELDI) technique. We report here the identification of five proteins that were differentially expressed in plaque and normal TA-derived cells.

2 Materials and methods

2.1 Cell culture

With the approval of our institutional review board and the consent of two patients, two pairs of TA tissue were obtained during venous grafting surgeries to correct the penile curvature [9]. Each pair consisted of a Peyronie's plaque and a piece of normal TA from the same patient. The tissues were washed three times in sterile phosphate-buffered saline (PBS) and cut into 2–3 mm^3 segments. The segments were placed evenly in a 100-mm cell culture dish (Falcon-Becton Dickinson Labware, Franklin Lakes, NJ, USA) inside a cell culture hood. Approximately 10 min later, 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing penicillin (100 units/mL), streptomycin (100 μg/mL), and 10 % fetal bovine serum (FBS) were carefully pipetted into the dish. The dish was then kept undisturbed in a humidified 37 °C incubator with 5 % CO_2. Five days later, tissue segments that had detached from the dish were removed, and the medium was replaced with fresh medium. Another 5 days later, all tissue segments were removed and the medium was again replaced with fresh medium. When small islands of cells were noticeable, they were trypsinized and transferred to a fresh culture dish. Expansion of each cell strain was continued with a change of medium every 3 days and passages (trypsinization and seeding) approximately every 10 days. All cells used in the following experiments were from passages 4 to 10.

2.2 Real-time polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated with a high pure RNA isolation kit (Roche Applied Science, Indianapolis, IN, USA). The RNA was reversely transcribed into cDNA as previously described [8], except that random primers (Invitrogen, La Jolla, CA, USA) were used in place of the poly-dT primer. The cDNA was then used for RT-PCR, using iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers for MCP-1 were (sense) 5'-GAGATCTGTGCTGACCCCAA-3' and (antisense) 5'-GACCCTCAAACATCCCAGG-3'. Primers for the glyceralde hyde-3-phosphate dehydrogenase (GAPDH) reference gene were (sense) 5'-ATTCCACCCATG-GCAAATTC-3' and (antisense) 5'-TGGGATTTCCAT-TGATGACAAG-3'. Cycling conditions were 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s and 55°C for 60 s, and finally 1 cycle at 95°C for 15 s, 55°C for 15 s and 95°C for 15 s. The results were analyzed with the SDS7000 software (Applied Biosystems, Foster City, USA). Quantification of MCP-1 expression was normalized to that of GAPDH.

2.3 Preparation of cellular protein lysates

Cells were cultured in 100-mm dishes to 80 % confluence. After the cells were washed with PBS, they were lysed in 250 μL of lysis buffer (25 mmol/L HEPES-KOH pH 7.4, 50 mmol/L KCl, 4 mmol/L $MgCl_2$, 1 % triton, 1 mmol/L PMSF) with three rounds of freeze-and-thaw in liquid nitrogen. After vigorous mixing and sitting on ice for 20 min, the lysate was centrifuged at 13,500 × *g* for 20 min at 4°C to pellet insoluble materials. The supernatant was measured for protein concentration by the

BCA method (Pierce Chemical Company, Rockford, IL, USA), and stored in aliquots of 20 μL at –80°C until use.

2.4 Fractionation of cell lysates

The crude cell lysate was fractionated using Ciphergen's Expression Difference Mapping Kit (Ciphergen, Fremont, CA, USA), which is capable of separating complex protein mixtures into 6 fractions largely on the basis of differences in the isoelectric point (pI). By this fractionation procedure, highly abundant proteins are segregated, thus reducing signal suppression effects on lower abundance proteins, and resulting in an increase of the number of peaks detected and of the probability of novel biomarkers being discovered.

2.5 Surface-enhanced laser desorption/ionisation time-of-flight (SELDI-TOF) analysis

We analyzed both crude and fractionated cell extracts with three different Ciphergen ProteinChips: IMAC30-Cu (metal affinity capture), CM10 (weak cation exchange), and H50 (reverse phase). After binding of proteins to the chips, sinapinic acid (SPA) was added as the energy absorbing molecule (EAM) to enable efficient laser desorption and ionization of proteins larger than 10 kDa. The chips were then transferred to the TOF mass spectrometer, and the instrument readout for each sample was visualized as a mass spectrum composed of peaks from 3 kDa to 100 kDa. The mass spectral pattern for each sample consisted of several peaks whose mass/charge ratios were depicted on the x-axis, while the height of the peak (relative abundance) was disposed along the y-axis.

2.6 Sample loading and washing

Cell lysates each containing 2.5 μg of protein were loaded in duplicate onto each spot in the protein chip. Before samples were analyzed by the SELDI-TOF MS, the loaded chips were washed twice using one of the following buffers: 100 mmol/L sodium phosphate (pH 7.0) for the IMAC30-Cu chip, 100 mmol/L sodium acetate for the CM10 chip, and 10 % acetonitrile/0.1 % trifluoroacetic acid (TFA) for the H50 chip. After binding of proteins to the chips, two aliquots of 1 μL of SPA in 50 % acetonitrile and 0.5 % TFA were added to each spot on the chip. The chips were then scanned in the TOF mass spectrometer for proteins from 10 kDa to 70 kDa. The laser intensity was adjusted depending on the signal-to-noise ratio of the mass peak heights to background. The spec-

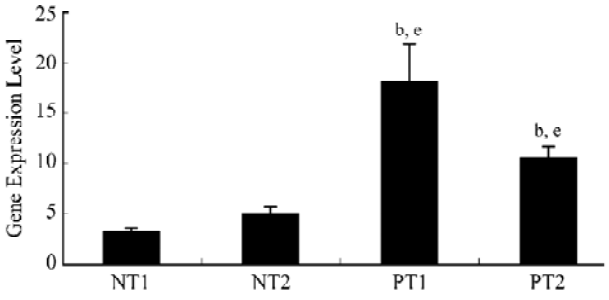

Figure 1. Elevated expression of MCP-1 in PT cells. Two plaque-derived cell strains (PT1 and PT2) and two normal tunica albuginea-derived cell strains (NT1 and NT2) were subjected to real-time polymerase chain reaction (RT-PCR) analysis. Each value is expressed as mean ± SD (n = 3). $^{b}P < 0.05$, compared with NT1, $^{e}P < 0.05$, compared with NT2.

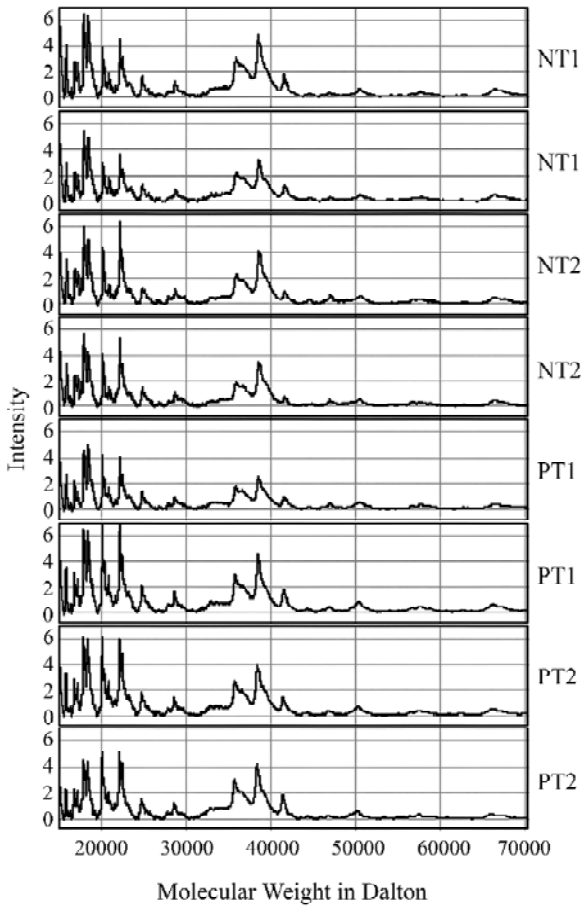

Figure 2. Reproducibility of SELDI-TOF MS in the proteomic analysis of crude cell lysates. Each lysate was analyzed twice on IMAC30-Cu chip. While the patterns obtained showed reproducibility for each lysate, they also indicated very similar profiles between different cell lysates.

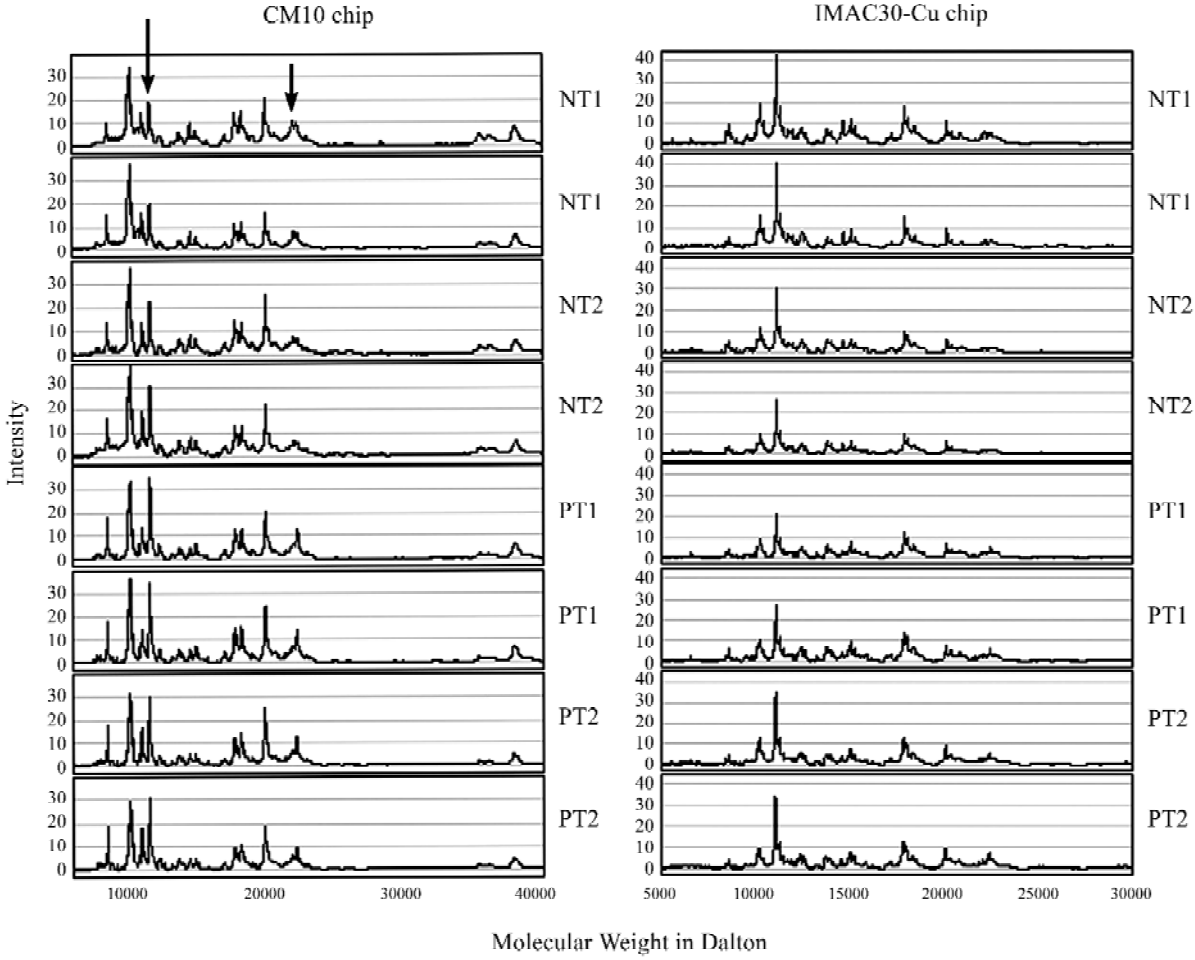

Figure 3. Analysis of fractionated proteins. Fraction-1 protein samples were analyzed on CM10 and IMAC30-Cu chips. The two downward arrows point to two protein species that were more abundantly expressed in PT than those in NT cells ($P < 0.05$).

tra were generated by using signals averaging 90 laser shots. Analysis of the mass spectra was performed using the ProteinChip software, version 3.2.0 (Ciphergen, Fremont, CA, USA). We utilized equine cardiac cytochrome C (13 kDa), equine cardiac myoglobulin (17 kDa), rabbit GAPDH (36 kDa), bovine serum albumin (66 kDa) and *Escherichia coli* beta-galacosidase (116 kDa) molecular weight standards as external calibrators.

3 Results

3.1 Establishment and validation of cell cultures

We previously reported the establishment of three sets of fibroblasts for the study of PD [8]. Each set included a Peyronie's plaque-derived P-cell strain, a "corner"-derived C-cell strain (semi-normal tunical cells), and a normal tunica-derived N-cell strain. These cells expressed increasing levels of monocyte chemoattractant protein-1 (MCP-1) from N- to C- to P-cells. While these cells have been proven useful, their phenotypes have become less stable because of increasing passage numbers. Therefore, for the present study we isolated new cell strains from two patients with Peyronie's disease. Cells from the plaque are called PT cells, and those from an unaffected area (normally-appearing tunica at corporotomy site at least 4 cm from the plaque) of the albuginea tunica are called NT cells. As expected, PT cells expressed higher levels of MCP-1 than NT cells (Figure 1).

3.2 SELDI-TOF MS

The crude cell lysates of the two pairs of PT and NT

cells were analyzed by SELDI-TOF on three ProteinChips of different chemistries. The protein spectrum generated for each cell strain on each ProteinChip was reproducible in two separate analyses. A representative result obtained with the IMAC30-Cu chip is shown in Figure 2.

Although the above mentioned data were reproducible, they did not sufficiently resolve differences between PT and NT cells, probably because of the crude nature of the protein samples. As such, in order to improve the sensitivity of detection, each crude cell lysate was separated into 6 fractions based largely on differences in the pI of proteins. These fractionated proteins were then analyzed on the three different protein chips. Shown in Figure 3 are profiles of fraction 1 (pH 9) of PT and NT cell lysates on CM10 and IMAC30-Cu chips, respectively. Further analyses of the protein profiles on the CM10 chip identified two protein species at 11.6 kDa and 22.6 kDa that were expressed at higher levels in PT than those in NT cells (Figures 4, 5).

Analysis of fraction 2 on IMAC30-Cu chip identified a 46.9-kDa protein that was less expressed in PT than that in NT cells (Figure 6). Analysis of fraction 3 on CM10 chip identified a 14.5-kDa protein expressed higher in PT than that in NT cells (Figure 7). Finally, analysis of fraction 4 on CM10 identified a 6.3-kDa protein less expressed in PT than that in NT cells (Figure 8).

4 Discussion

Cells derived from Peyronie's plaque (PT cells) exhibit unique morphology and growth characteristics [6–8]. However, the genetic basis for these distinct properties remains poorly understood. Recently we reported that the fibrogenic cytokine MCP-1 was more abundantly expressed in PT than that in NT cells, implicating its involvement in the fibrogenesis of tunical cells during the progression of PD. In the present study, we first showed that two newly established PT cell strains also

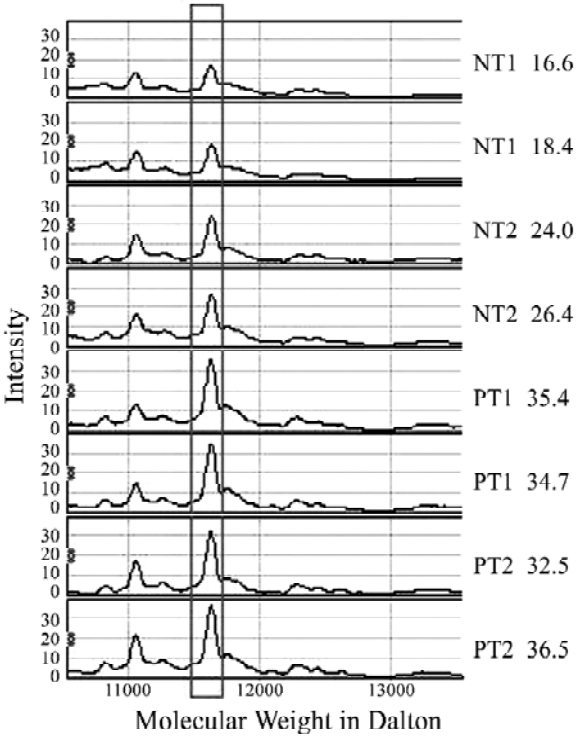

Figure 4. Identification of an upregulated 11.6-kDa protein in PT cells. Analysis of fraction-1 protein samples on CM10 chip revealed an 11.6-kDa protein that was more abundantly expressed in PT than that in NT cells ($P < 0.05$). Numbers on the rightmost column are intensities of the 11.6-kDa peaks.

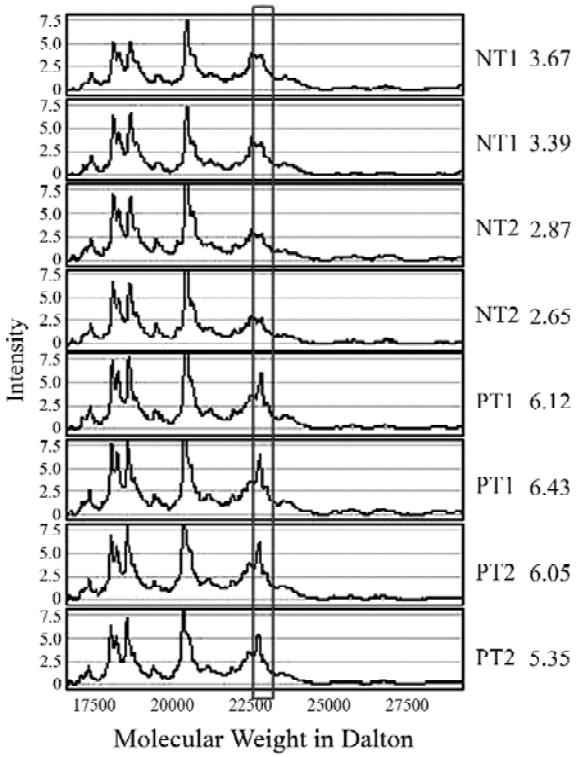

Figure 5. Identification of an upregulated 22.6-kDa protein in PT cells. Analysis of fraction-1 protein samples on CM10 chip revealed a 22.6-kDa protein that was more abundantly expressed in PT than that in NT cells ($P < 0.05$). Numbers on the rightmost column are intensities of the 22.6-kDa peaks.

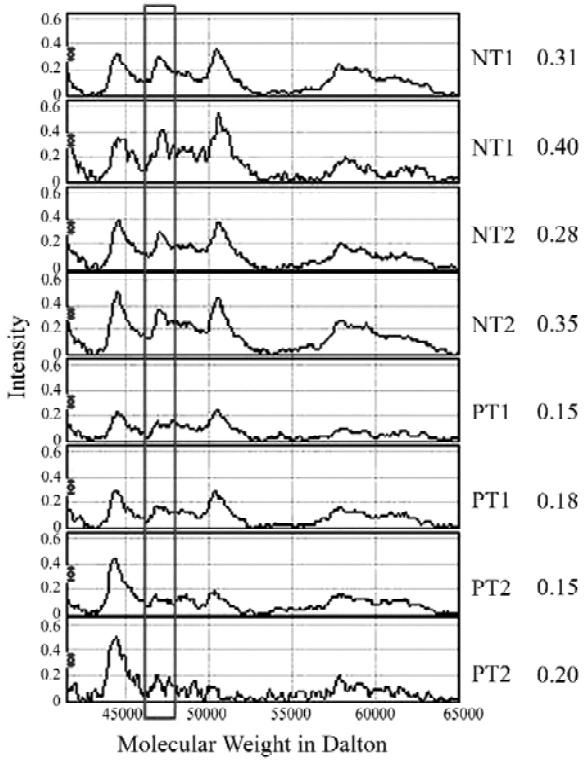

Figure 6. Identification of a downregulated 46.9-kDa protein in PT cells. Analysis of fraction-2 protein samples on IMAC30-Cu chip revealed a 46.9-kDa protein that was less abundantly expressed in PT than that in NT cells ($P < 0.05$). Numbers on the rightmost column are intensities of the 46.9-kDa peaks.

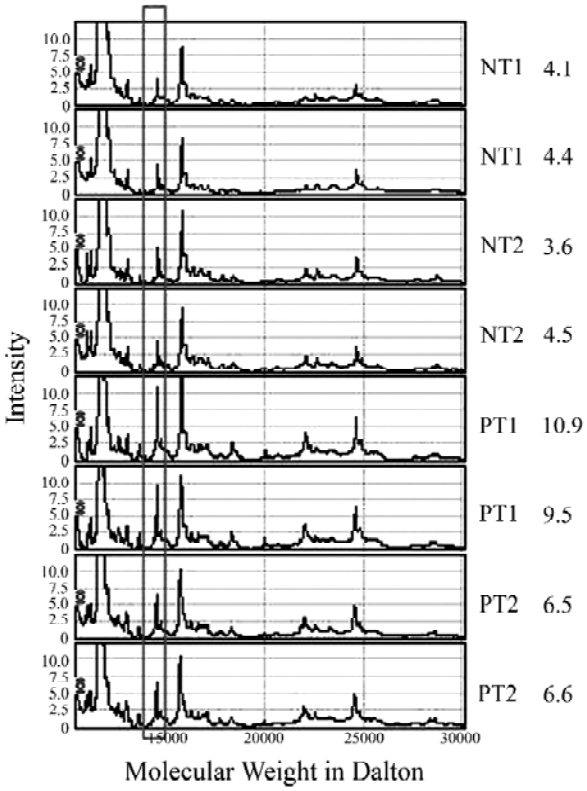

Figure 7. Identification of an upregulated 14.5-kDa protein in PT cells. Analysis of fraction-3 protein samples on CM10 chip revealed a 14.5-kDa protein that was more abundantly expressed in PT than that in NT cells ($P < 0.05$). Numbers shown on the rightmost column are intensities of the 14.5-kDa peaks.

expressed higher levels of MCP-1 than their counterpart NT cell strains. These results not only strengthened the MCP-1/PD connection but also validated the new cell strains for further analyses. We then subjected these cell strains to SELDI-TOF MS analyses, which were aimed at identifying protein markers that are differentially expressed in normal and diseased TA. Initial analyses of the crude cellular protein lysates revealed a high degree of similarity among all four cell strains, indicating that: 1) the cell strains were indeed derived from a common tissue source – the TA of the penis, 2) the protein lysates were of comparable quality, and 3) the protein chips possessed consistent and reproducible properties..

To improve the chance of identifying subtle yet more relevant changes in protein expression among the cell strains, we separated each crude protein lysate into six different fractions based on the differences in pI of the proteins. Analyses of these fractionated proteins with the protein chips resulted in the identification of three upregulated and two downregulated proteins in PT cells. Two of the upregulated proteins have molecular weights of 11.6 kDa and 14.5 kDa, respectively, which are close to the estimated molecular weights of the glycosylated forms of human MCP-1. Further investigation is needed to reveal the identities of these differentially expressed proteins.

In conclusion, upregulated MCP-1 expression was reproducibly observed in PT cells. SELDI-MS analyses identified five potential biomarkers for PD: three of 11.6 kDa, 14.5 kDa and 22.6 kDa were upregulated, while two of 6.3 kDa and 46.9 kDa were downregulated.

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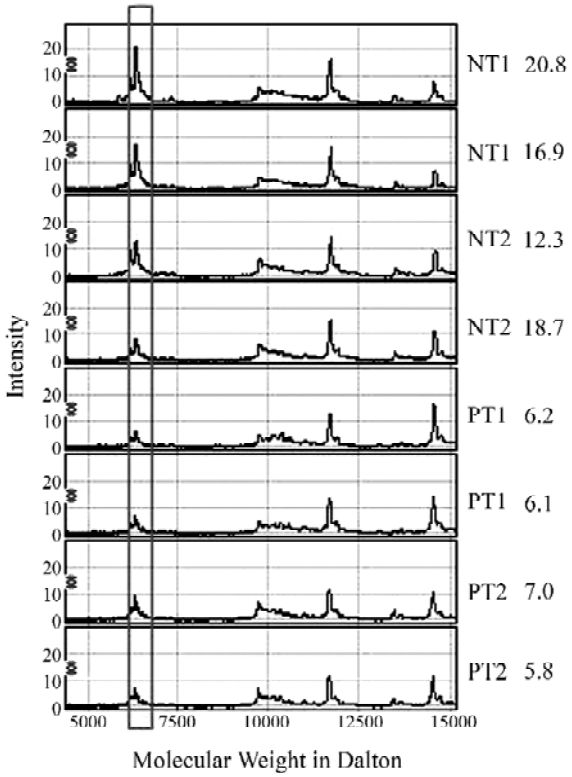

Figure 8. Identification of a downregulated 6.3-kDa protein in PT cells. Analysis of fraction-4 protein samples on CM10 chip revealed a 6.3-kDa protein that was less abundantly expressed in PT than that in NT cells ($P < 0.05$). Numbers shown on the rightmost column are intensities of the 6.3-kDa peaks.

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