ANTICANCER RESEARCH *25*: 1197-1204 (2005)

Identification of Serum Proteins Related to Adverse Effects Induced by Docetaxel Infusion from Protein Expression Profiles of Serum Using SELDI ProteinChip System

YUJI HEIKE[1,2,3], MAMI HOSOKAWA[1,2], SHOZO OSUMI[1], DAISUKE FUJII[1], KENJIRO AOGI[1], NAGIO TAKIGAWA[1], MIKIKO IDA[1], HISAO TAJIRI[1], KENJI EGUCHI[1], MIEKO SHIWA[4], RUMI WAKATABE[4], HISASHI ARIKUNI[5], YOICHI TAKAUE[2] and SHIGEMITSU TAKASHIMA[1]

[1]*Department of Medical Oncology and Clinical Research, National Shikoku Cancer Center, 13-Horinouchi, Matsuyama, Ehime 790-0007;*
[2]*Hematopoietic Stem Cell Transplant/Immunotherapy Unit, National Cancer Center Hospital, Tsukiji, Chuo-ku, Tokyo 104-0045;*
[3]*Pharmacology Division, National Cancer Center Research Insitute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045;*
[4]*Ciphergen Biosystems K.K., Yokohama Business Park Hi-tech Center 1F, 134 Godo-cho, Hodogaya-ku, Yokohama-shi, Kanagawa, 240-0005;*
[5]*Sumitomo-Bioscience, Yokohama Business Park Hi-tech Center 1F, 134 Godo-cho, Hodogaya-ku, Yokohama-shi, Kanagawa, 240-0005, Japan*

Abstract. *Background: For the development of quick and easy methods for screening and identifying treatment-responsive proteins, we determined the protein expression profile of the serum after docetaxel infusion using a surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI TOF-MS) system. Materials and Methods: Blood from breast cancer patients was collected before and 4, 8, 24 and 48 hours after docetaxel infusion. The protein expression profile was determined by a SELDI TOF-MS system. The relative expression levels of target proteins were compared during the time-course after docetaxel injection. Results: We identified two representative proteins with molecular weights of 7790 Da and 9285 Da. The 7790 Da protein was high molecular weight kininogen, and the 9285 Da protein was apolipoprotein A-II. These two proteins had similar expression patterns in 5 patients, except one patient who experienced severe, acute, adverse effects. Conclusion: These results suggest that protein expression profiles determined by SELDI TOF-MS represent useful data for the identification of treatment-responsive proteins.*

Correspondence to: Yuji Heike, MD, PhD, Pharmacology Division, National Cancer Center Research Insitute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Tel: +81-3-3542-2511, Fax: +81-3-3547-5228, e-mail: yheike@ncc.go.jp

Key Words: SELDI TOF-MS, protein expression profile, adverse effect, ProteinChip System, HMW kininogen, apolipoprotein A-II.

Docetaxel is a key drug used to treat breast cancer. As docetaxel is hydrophobic, polysorbate 80 and ethanol are used as solvents. Some patients experience acute adverse effects, including anaphylactoid reactions, during docetaxel infusion and may develop shock. Several reports suggest that these adverse effects are caused by the solvent, which contains polysorbate 80 (1, 2). However, the mechanisms of these adverse effects are unknown. One approach to determine the mechanisms causing adverse effects is to analyze the treatment-responsive proteins.

Pharmacokinetics and pharmacodynamics are important areas of research in clinical pharmacology that analyze drug metabolism and host responses in order to predict the response and adverse effects of treatment.

Recently, DNA chip technology, such as DNA microarrays and cDNA arrays, has been used to predict responses to treatment. Some promising results have been reported in studies of irinotecan and other drugs (3-5), and there is no doubt that this genetic approach could be used to predict the potential response of a patient to treatment. However, genetic information alone cannot perfectly predict responses to treatment, because genes do not act by themselves. Genes exert their effect through proteins after transcription and translation. Thus, protein analysis is important to predict responses to treatment.

Until recently, no ideal tools have been available to determine protein expression profiles. Although two-dimensional electrophoresis could be used for this purpose, it is labor-intensive and is not capable of high-throughput

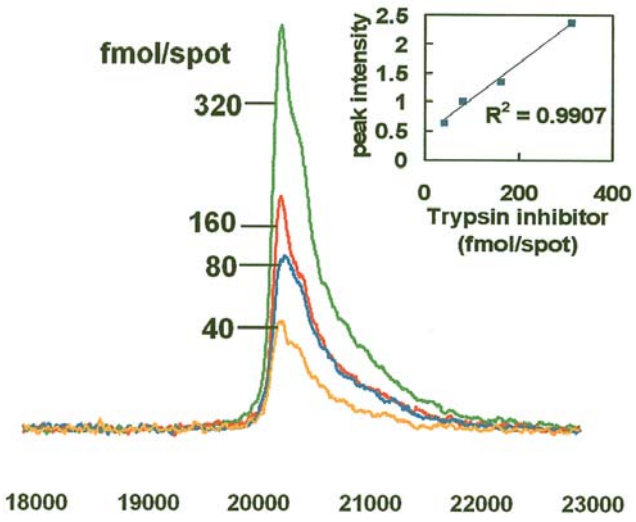

Figure 1. *Quantitative analysis. Various concentrations of trypsin inhibitor were studied with the SAX-2 (strong anion exchange) chip. Peak heights of trypsin inhibitor (molecular weight, approximately 20,000 Da) were clearly dose-dependent. The correlation coefficient calculated by Excel 2000 software was 0.99.*

analysis. Recent developments in mass spectrometry have been revolutionary in proteomic research. These new techniques are highly sensitive and have potential for various applications. Surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI TOF-MS) can be used to produce protein expression profiles. This system comprises ProteinChip arrays and time-of-flight mass spectrometry (TOF-MS). ProteinChip arrays possess varying chromatographic properties, such as anion exchange, cation exchange, metal affinity and reverse phase. The combination of the type of chip array and the washing conditions, such as the pH or salt concentration in the buffer, allows rapid analysis of protein profiles with only a small amount of sample. In the present study, we used the SELDI TOF-MS system to find docetaxel treatment-responsive proteins.

To select treatment-responsive proteins, it was necessary to compare the protein expression levels at several time-points after the treatment. However, no previous studies have indicated that mass spectrometry can be used for quantitative analysis of proteins. In the present study, we conducted a quantitative analysis of the targeted proteins. After selecting two representative treatment-responsive proteins, we used SELDI TOF-MS to determine the conditions for column purification of the proteins. We then determined the amino acid sequence of the purified proteins. The relationship between these proteins and docetaxel-induced shock is discussed, as well as the usefulness of the present approach in clinical pharmacology and clinical proteomics.

Table I.

Research ID	Gender	Protocol	Acute response
A	F	tri-week (60mg/m^2)	No
B	F	tri-week (60mg/m^2)	No
C	F	tri-week (60mg/m^2)	No
D	F	tri-week (60mg/m^2)	Shock
E	F	weekly (40mg/m^2)	No

Materials and Methods

Quantitative analysis. Various concentrations of trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) were studied using the SAX-2 chip (Ciphergen Biosystems, Fremont, CA, USA), and the heights of peaks were compared for the quantitative analysis.

Patients and blood samples. Ten breast cancer patients receiving docetaxel treatment were enrolled in this experiment, after providing informed consent in accordance with the guidelines of the National Shikoku Cancer Center Institutional Review Board, Japan. The results from 6 representative patients are described in the present report. Patients receiving docetaxel injections (60 mg/m^2) every 3 weeks and patients receiving weekly docetaxel injections (40 mg/m^2) were enrolled in this study. Dexamethasone (8 mg) was used as a premedication 30 min prior to docetaxel infusion. Docetaxel was administered as a 30-min infusion for patients on the weekly schedule and as a 60-min infusion for patients on the 3-week schedule. After the docetaxel infusions, oral dexamethasone (4 mg) was taken twice daily for one day for patients on the weekly schedule and for 2 days for patients on the 3-week schedule.

Blood (5 ml) was collected before docetaxel infusion and 4, 8, 24 and 48 h after docetaxel infusion. Serum was prepared quickly and stored at -80˚C until analysis.

Medical information, such as adverse effects after docetaxel injection, was obtained from the medical records and entered into a database in accordance with the privacy policy of our institution.

Protein expression profiles. Protein expression profiles were determined using a SELDI TOF-MS system (Ciphergen Biosystems). IMAC3 (immobilized metal affinity capture), WCX-2 (weak cation exchange) and SAX-2 (strong anion exchange) ProteinChip arrays were used for analysis. The serum samples were centrifuged at 12,000 rpm using a microcentrifuge (TOMY Tech USA, Fremont, CA, USA). The supernatant was vigorously mixed with urea buffer (8 M urea:1% CHAPS/PBS, 1:1) for 10 min at 4˚C and was diluted with $\frac{5x}{1x}$ volumes of binding buffer (PBS for IMAC3, 50 mM phosphate buffer (pH 6) for WCX-2 and 50 mM Tris-HCl (pH 8) for SAX-2).

To immobilize copper onto the IMAC3 surface, 5 μl of 50 mM copper sulfate was loaded, and the chip was shaken for 5 min. Excess copper was removed under running deionized water, and the chip was shaken for 5 min with 10 μl of 50 mM sodium acetate (pH 4). The chip was rinsed under running deionized water and was then ready to be used for the analysis step.

The following procedure was used for chip analysis: (i) each spot was equilibrated with 150 μl of binding buffer twice on a shaker for 5 min, and excess buffer was removed; (ii) diluted samples (50 μl per

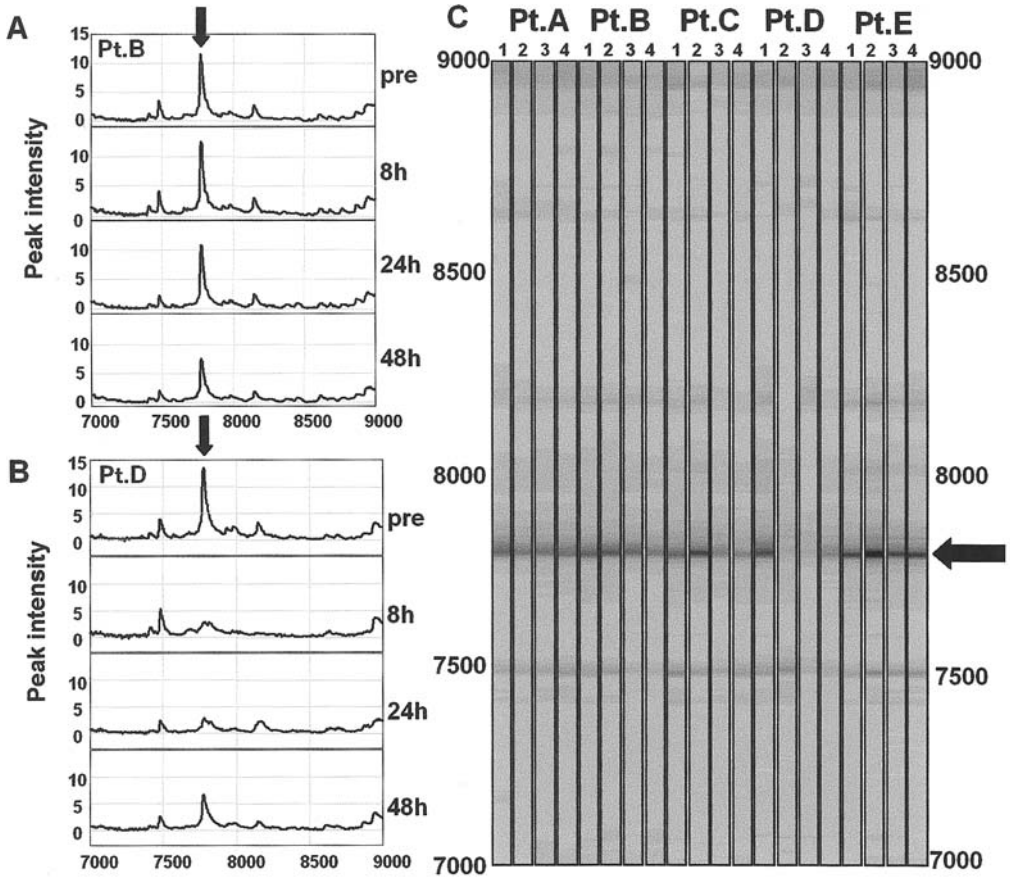

Figure 2. *Expression of 7790 Da protein at different times. (A) Typical response pattern of the 7790 Da protein in patient B, in trace view. (B) Specific response pattern in patient D, in trace view. (C) Gel view of 5 samples (Pt. A-Pt. E). Lane 1, pre-injection; lane 2, 8 h after injection; lane 3, 24 h after injection; and lane 4, 48 h after injection. In patient D, the protein suddenly disappeared and slowly reappeared over a period of days.*

spot) were loaded, and the chip was incubated on a shaker for 20 min at room temperature; (iii) the chip was washed 3 times with 150 μl per spot of binding buffer; (iv) the chip was rinsed with distilled water and dried; and (v) each spot was treated with 0.5 μl of saturated sinapinic acid prepared in aqueous solution containing 50% acetonitrile and 0.5% trifluoroacetic acid. Captured proteins were directly detected using a PBS II ProteinChip Reader (Ciphergen Biosystems).

Screening of docetaxel-responsive proteins. Quantitative analysis of proteins was performed using Peaks 3.0 software (Ciphergen Biosystems). Expression levels of the proteins were compared over the time-course of the experiment. Two representative proteins, that had similar expression patterns in patients, were selected.

Protein purification and amino acid sequencing. To determine the optimal pH and salt concentration of the buffer for purification of the target proteins, IMAC3, WCX-2 and SAX-2 assays were performed. The target proteins were fractionated by CM Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ, USA) and separated by 16% SDS polyacrylamide gel electrophoresis using the method reported by Schagger and Jagow (6). Electroblotting to PVDF membrane was performed using a TEFCO electroblotting system (TEFCO. Co., Ltd., Tokyo, Japan). Amino acid sequences of the purified proteins were analyzed according to the Edman method using a Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA, USA). Database searches of the sequence data were performed using SWISS-PROT.

Results

Quantitative analysis. To confirm that the SELDI TOF-MS system can be used for quantitative analysis, various concentrations of trypsin inhibitor were analyzed using the SAX-2 chip. The peak heights of trypsin inhibitor (molecular weight, approximately 20,000 Da) occurred in a dose-dependent manner (Figure 1) with a correlation coefficient of 0.99 calculated by Excel 2000 software. The SELDI TOF-MS system can therefore be used to quantitatively analyze protein profiles.

Patient characteristics and acute reaction related to docetaxel injection. Ten patients were enrolled in the study, and the data from 6 representative patients are presented in this report. The background of the patients, injection schedule and acute reactions recorded in the medical records are

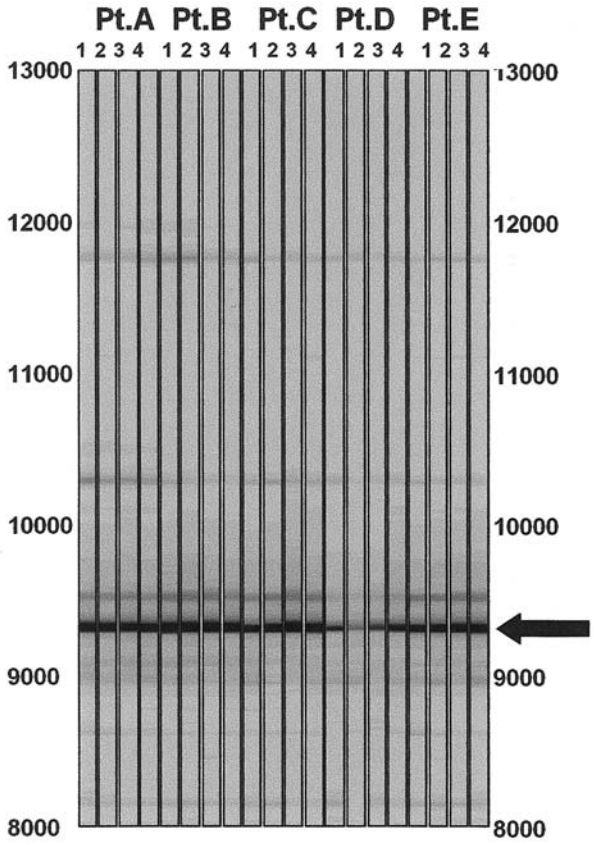

Figure 3. *Gel view of 9285 Da protein in 5 patients. In patient D, expression of the 9285 Da protein decreased transiently. The expression of this protein was unchanged in all other patients. Lane 1, pre-injection; lane 2, 8 h after injection; lane 3, 24 h after injection; and lane 4, 48 h after injection.*

summarized in Table I. Five of the 6 patients experienced no severe adverse effects, although slight flushing of the face was observed in two patients. Only one patient (patient D) experienced severe, acute, adverse effects and experienced signs and symptoms of shock. Severe flushing of the face, tachypnea with dyspnea, tachycardia and low blood pressure were observed in patient D. The docetaxel injection was stopped immediately and additional dexamethasone (8 mg) was injected. The patient recovered from shock, and docetaxel dissolved in simple saline (without polysorbate 80) was slowly infused. The infusion was completed without any additional adverse effects.

Protein profiles and docetaxel-responsive proteins. We compared serial profiles from the same patients according to the time-course of the infusion and selected two peaks of interest. The peaks occurred at 7790 Da and 9285 Da. Figure 2A shows data from patient B, and Figure 2B shows data from patient D. Figure 2C shows data from 5 patients and indicates that the expression of the 7790 Da protein disappeared suddenly only in patient D. Patient D, who

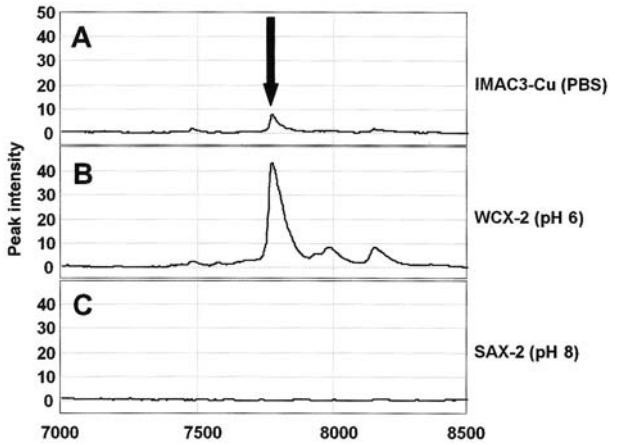

Figure 4. *Binding capacity of 7790 Da protein to three different chips. (A) Immobilized metal affinity capture using copper (IMAC3-Cu chip, PBS buffer), (B) weak cation exchange (WCX-2 chip, pH 6 buffer) and (C) strong anion exchange (SAX-2 chip, pH 8 buffer). The signal obtained from the WCX-2 chip displayed the highest intensity.*

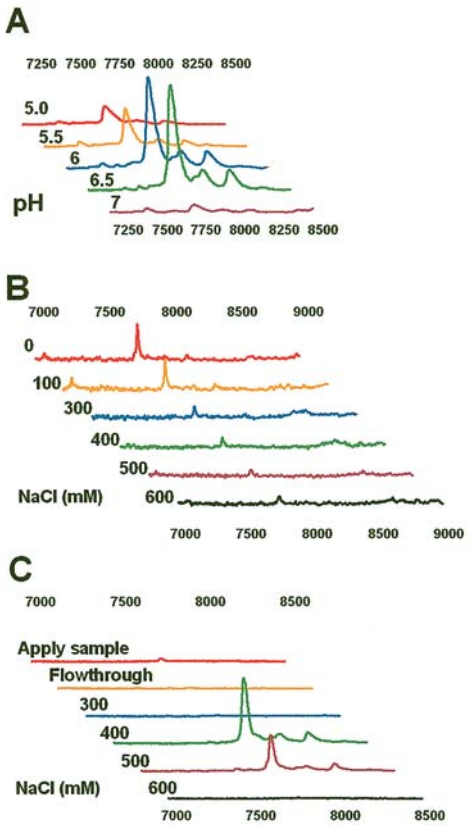

Figure 5. *Capture and elution conditions for 7790 Da protein. (A) Capture pH for the 7790 Da protein using the WCX-2 chip. (B) NaCl concentration for elution of the 7790 Da protein from the WCX-2 chip. (C) NaCl concentration for purification of the 7790 Da protein by CM sepharose affinity chromatography. These results suggest that the best conditions were pH 6 for capture and 300-400 mM NaCl for elution from the WCX-2 chip. In CM sepharose columns, buffer at pH 6 was used as a capture solution, and the optimal concentration of NaCl for elution was 400 mM.*

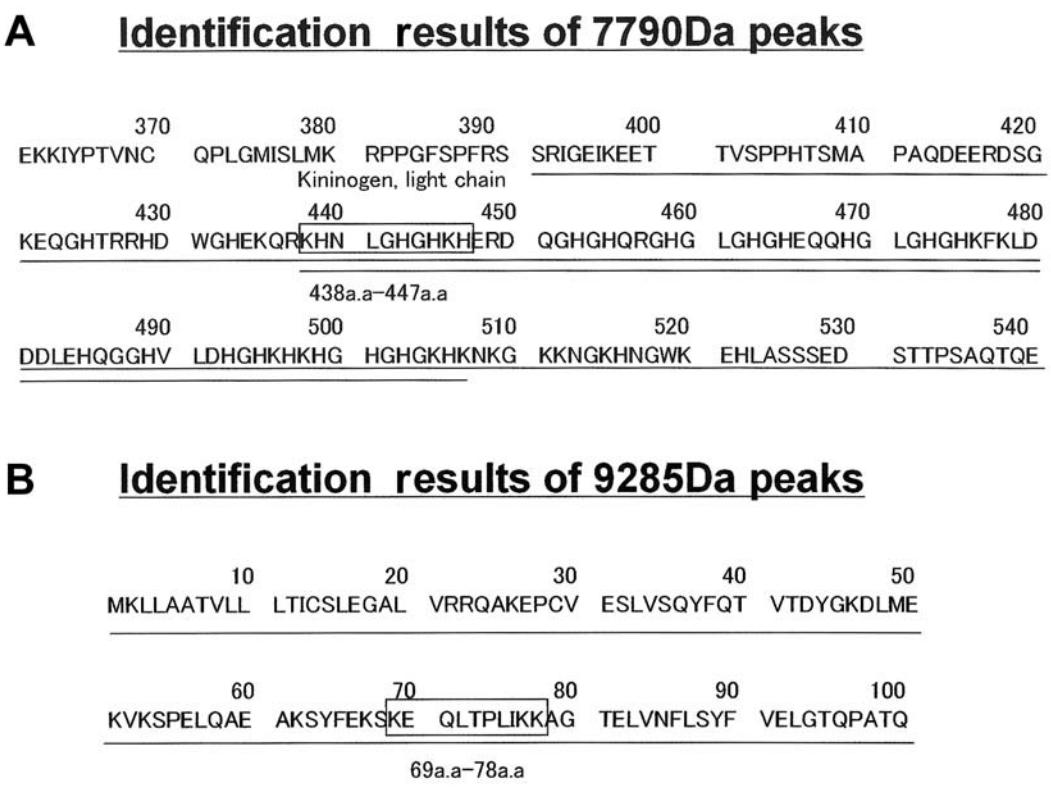

Figure 6. *Amino acid sequences of target proteins. (A) Ten amino acids from the NH3 terminal of the 7790 Da protein were analyzed according to the Edman method. The sequence was identical to amino acids 438-447 of high molecular weight kininogen. (B) Ten amino acids from a fragment of the 9285 Da protein were analyzed after in-gel digestion. The sequence was identical to amino acids 69-78 of apolipoprotein A-II.*

experienced severe adverse effects during docetaxel injection, displayed a rapid down-regulation of the 7790 Da protein, but the expression of this protein recovered over several days. Figure 3 shows gel view data of the 9285 Da protein from the same samples. The expression pattern of this protein was almost the same as the 7790 Da protein.

Purification of target proteins. To establish a procedure to purify the target proteins, we determined which chip is preferable to capture the target proteins, and then determined the optimal pH and salt concentration in the buffer using protein arrays. The results revealed that both proteins bound to the WCX-2 chip more strongly than to the other two chips (data from 7790 Da protein shown in Figure 4).

The capture pH and the NaCl concentration for elution of the 7790 Da protein were investigated using the WCX-2 chip (Figure 5). The results indicated that the best conditions were pH 6 for capture (Figure 5A) and 300-400 mM NaCl for elution (Figure 5B). We used these conditions in large-scale purification using CM sepharose column chromatography. We diluted 11.4 ml of serum to 100 ml with sodium phosphate and citrate buffer (pH 6) and applied it to a CM sepharose column (25 cm). After equilibrating with the same buffer, elution was performed using 300-600 mM NaCl stepwise. The target protein was eluted under the conditions indicated by the protein chip analysis (Figure 5C). After the 300-400 mM NaCl fraction had been dialyzed and concentrated, the sample was applied to SDS PAGE. The band containing the 7790 Da protein was removed from the gel. The purification procedure for the 9285 Da protein was determined by almost the same process, and the protein was purified (data not shown).

Amino acid sequencing of the target proteins. To determine the amino acid sequence of the target proteins, the proteins were transferred from a gel to PVDF membranes. The amino acid sequence of the purified protein was determined according to the Edman method. A sequence of 10 amino acids from the NH3 end of the 7790 Da protein was directly analyzed and determined to be identical to amino acids 438-447 of high molecular weight (HMW) kininogen (Figure 6A). This protein was also analyzed by simple MS and MSMS system, and was identified as kininogen (Figure 7). The amino terminal end of the 9285 Da protein was blocked and could not be analyzed directly. Analysis of the amino acid sequence was therefore performed after tryptic digestion, and a 10 amino acid internal sequence from the

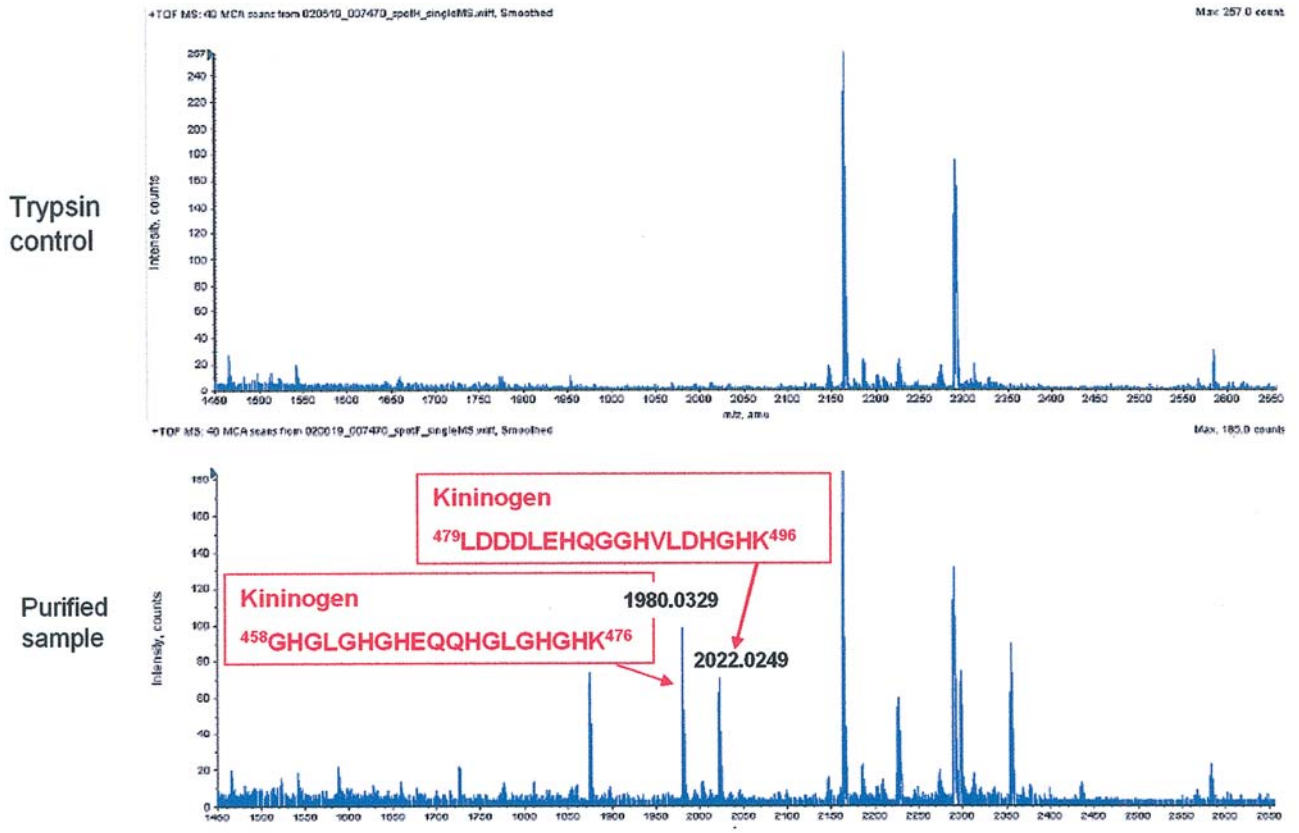

Figure 7. *Single MS and MSMS analysis of tryptic in-gel digestion sample. A piece of the gel containing 7790 Da proteins was treated with trypsin and the digested peptides were analyzed by MS and MSMS systems. Typical signals, which were considered to be fragments of kininogen, were detected.*

9285 Da protein was determined. The sequence was identical to amino acids 69-78 of apolipoprotein A-II (Figure 6B).

Discussion

Widespread expression analysis of DNA became available with the development of cDNA microarrays. Predictions of response and adverse effects were recently studied using DNA microarrays and cDNA expression arrays. However, it is clear that DNA and mRNA do not work by themselves, because mRNA must be translated into protein. This principle indicates the importance of protein analysis in post-genome projects. However, no ideal tool was previously available to determine protein expression profiles. Although two-dimensional electrophoresis could be used in this respect, the process is labor intensive and incapable of high-throughput analysis. In addition, two-dimensional electrophoresis requires skillful techniques to obtain reproducible data.

Mass spectrometry techniques have recently been used in the field of protein analysis. Liquid chromatography combined with TOF-MS offers very high sensitivity. However, this system cannot produce protein expression profiles. SELDI TOF-MS, which was recently developed, can determine protein expression profiles from crude samples, such as serum, urine and other body fluids. SELDI TOF-MS is constructed with TOF-MS and chip arrays, which have chromatographic properties on their surface. SELDI TOF-MS can be used to quickly develop protein profiles using only a small amount of sample. Several groups have found biological markers such as tumor markers using this system (7-13).

In the present study, we developed protein expression profiles from serum collected before and after docetaxel injection. We compared the protein expression profiles over the time-course of the infusion to find docetaxel-responsive proteins. We determined the amino acid sequences of the selected proteins. One of the docetaxel-responsive proteins was HMW kininogen, which is a factor in the kinin/kallikrein cascade of blood coagulation. This protein suddenly decreased in patient D, who experienced severe shock during docetaxel injection. No other patients displayed sudden decreases in this protein. Cochrane *et al.* reported a relationship between HMW kininogen and hypotensive shock (14), and Gallimore reported changes in HMW kininogen during lethal endotoxin shock (15). These reports suggest that HMW kininogen is a shock-related protein, although no previous reports have suggested that HMW kininogen is related to drug-induced

shock. The other docetaxel-responsive protein that we detected was apolipoprotein A-II, which is related to lipid and cholesterol metabolism. To the best of our knowledge, no previous reports have described an association between apolipoprotein A-II and shock.

The expression levels of HMW kininogen and apolipoprotein A-II in all patients (except for patient D) were unchanged or were slightly decreased and recovered quickly without treatment. No severe adverse effects were observed in these patients, although some experienced slight flushing of the face. These findings suggest that the homeostatic mechanisms of the body worked quickly, allowing these patients to recover from the effects of docetaxel infusion.

The roles of the proteins detected in our study in docetaxel-induced shock are unknown. Even if the changes in these proteins are the result of shock, rather than the cause of shock, this information may help elucidate the mechanisms of docetaxel-induced shock and may lead to measures for prediction of and protection from shock. Our ultimate goals are to find predictive markers for adverse effects in order to prevent/treat adverse effects, and to find molecular targets of the response. We have started to screen other proteins that respond to docetaxel treatment in order to identify proteins causing adverse effects.

Two noteworthy technical points from our experiments have not previously been reported. The first point is that we successfully used SELDI TOF-MS to compare protein expression levels in crude samples, which is an important step for screening responsive proteins. Previously, clear evidence that mass spectrometry can be used for quantitative analysis of protein from crude samples has been unavailable. Therefore, the SELDI ProteinChip System may be a key technology for protein expression analysis. The ProteinChip System can also perform high-throughput analysis. This technology is very useful for the discovery of biomarkers, particularly when a large number of samples need to be analyzed. The second noteworthy technical point is that we determined the procedure for protein purification in only one day using SELDI TOF-MS. The purification conditions determined by SELDI TOF-MS could be applied to large-scale column chromatography for both proteins. These results indicate that high-speed purification is possible using SELDI TOF-MS.

In conclusion, we found that the SELDI TOF-MS ProteinChip System can be used to compare protein expression levels in protein profiles and to quickly purify target proteins. These two points may lead to breakthroughs in clinical proteomics and clinical pharmacology.

Acknowledgements

This work was supported by grants from the Institute for Clinical Research, Department of National Hospitals, Ministry of Health, Labour and Welfare of Japan.

References

1 Rhodes A, Eastwood JB and Smith SA: Early acute hepatitis with parenteral amiodarone: a toxic effect of the vehicle? Gut *34*: 565-566, 1993.
2 Battafarano DF, Zimmerman GC, Older SA, Keeling JH and Burris HA: Docetaxel (Taxotere) associated scleroderma-like changes of the lower extremities. A report of three cases. Cancer *76*: 110-115, 1995.
3 Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K and Hasegawa Y: Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res *60*: 6921-6926, 2000.
4 Ando Y, Ueoka H, Sugiyama T, Ichiki M, Shimokata K and Hasegawa Y: Polymorphisms of UDP-glucuronosyltransferase and pharmacokinetics of irinotecan. Ther Drug Monit *24*: 111-116, 2002.
5 Ando M, Ando Y, Sekido Y, Shimokata K and Hasegawa Y: Genetic polymorphisms of the UDP-glucuronosyltransferase 1A7 gene and irinotecan toxicity in Japanese cancer patients. Jpn J Cancer Res *93*: 591-597, 2002.
6 Schagger H and von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem *166*: 368-379, 1987.
7 Davies H, Lomas L and Austen B: Profiling of amyloid beta peptide variants using SELDI Protein Chip arrays. Biotechniques *27*: 1258-1261, 1999.
8 Paweletz CP, Trock B, Pennanen M, Tsangaris T, Magnant C, Liotta LA and Petricoin EF 3rd: Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: potential for new biomarkers to aid in the diagnosis of breast cancer. Disease Markers *17*: 301-307, 2001.
9 Rubin RB and Merchant M: A rapid protein profiling system that speeds study of cancer and other diseases. Am Clin Lab *19*: 28-29, 2000.
10 Verma M, Wright GLJ, Hanash SM, Gopal-Srivastava R and Srivastava S: Proteomic approaches within the NCI early detection research network for the discovery and identification of cancer biomarkers. Ann NY Acad Sci *945*: 103-115, 2001.
11 Ardekani AM, Liotta LA and Petricoin EF 3rd: Clinical potential of proteomics in the diagnosis of ovarian cancer. Expert Rev Mol Diagn *2*: 312-320, 2002.
12 Issaq HJ, Veenstra TD, Conrads TP and Felschow D: The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun *292*: 587-592, 2002.
13 Diamandis EP: Proteomic patterns in serum and identification of ovarian cancer. Lancet *360*: 170-171, 2002.
14 Cochrane CG and Revak SD: The participation of high molecular weight kininogen in hypotensive shock and intravascular coagulation. Clin Immunol Immunopathol *15*: 367-374, 1980.
15 Gallimore MJ, Aasen AO and Amundsen E: Changes in plasma levels of prekallikrein, kallikrein, high molecular weight kininogen and kallikrein inhibitors during lethal endotoxin shock in dogs. Haemostasis *7*: 79-84, 1987.

Received August 9, 2004
Accepted January 27, 2005