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Original Article

Assessment of Protein Stability in Cerebrospinal Fluid Using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Protein Profiling

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Abstract

Recent studies have evaluated proper acquisition and storage procedures for the use of serum or plasma for mass spectrometry (MS)-based proteomics. The present study examines the proteome stability of human cerebrospinal fluid (CSF) over time at 23°C (room temperature) and 4°C using surface-enhanced laser desorption/ionization time-of-flight MS. Data analysis revealed that statistically significant differences in protein profiles are apparent within 4 h at 23°C and between 6 and 8 h at 4°C. Inclusion of protease and phosphatase inhibitor cocktails into the CSF samples failed to significantly reduce proteome alterations over time. We conclude that MS-based proteomic analysis of CSF requires careful assessment of sample collection procedures for rapid and optimal sample acquisition and storage.

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Introduction

Biomarker discovery is at the forefront of biomedical research, and mass spectrometry (MS)-based clinical proteomics has become a popular method to both streamline drug discovery and provide specific diagnostic biomarkers and surrogate end points for a number of human diseases (1–7). However, the ability of MS (or other proteomic techniques) to accurately characterize the proteome is dependent on the extent to which the clinical samples were appropriately handled and stored to preserve protein and peptide integrity. Clinical samples are often initially stored either at room temperature or on ice for various lengths of time before being received by a research laboratory for final sample preparation and long-term storage. Prior studies have shown that sample handling and storage conditions are critical variables in protein analysis of blood serum, urine, and tissue samples (8–12). Another study demonstrated that reproducible and reliable measurements of apolipoprotein E within the cerebrospinal fluid (CSF) were dependent on consistent sample handling and storage conditions (13).

Many ongoing proteomic biomarker discovery and validation studies are collaborative multicenter projects, which can confound efforts to apply consistent sample acquisition and storage conditions, especially if samples are obtained through surgical procedures. Any preanalytical errors or procedural differences between the various participant sites, or even the adoption of uniform but suboptimal, untested platform standardization strategies, will ultimately affect the final data analysis and potentially lead to erroneous identification of "artifact" biomarkers (14,15). It is, therefore, crucial to characterize the impact of sample collection, handling, and storage procedure and identify best laboratory practices to reduce experimental variation and enhance quality control, even at the biomarker discovery stage.

Despite many clinical proteomic studies using CSF (4,16–18), there are no reports on the stability of the CSF proteome over time when incubated at various temperatures. A prior study identified instabilities of CSF metabolites by proton nuclear magnetic resonance after 72-h exposure to room temperature (19). A number of age-related changes in the CSF proteome have been recently reported; an important consideration for age-dependent disease processes, such as neurodegenerative disorders (20). Temperature storage-related artifacts have also been reported for the cystatin C protein within human CSF (15). Prefractionation of CSF via chromatography or gel electrophoresis has also been used to analyze its proteomic content (21). Recent investigations have also examined proper collection and storage procedures for plasma proteomics using two-dimensional (2D) gels or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS (22,23), or highlighted factors affecting the reproducibility of protein profiling in urine many months after initial examination (3). In addition, a recent study evaluated the presence of blood protein contaminants in the CSF using 2D gels and determined that CSF proteins of blood contaminated samples were less stable than CSF proteins with no blood contamination (24). A current report has demonstrated the reproducibility of the surface-enhanced laser desorption/ionization (SELDI)-TOF-MS protein profiling system across multiple laboratories using human serum (14). The aim of the current study was to evaluate the stability of the CSF proteome to variations of temperature and time, as determined by SELDI-TOF-MS protein profiling.

Methods

Samples

CSF was obtained by lumbar puncture from four healthy adults (three female, one male, age range from 17 to 65 yr) on University of Pittsburgh approved institutional review board consent. Subject 1 is a 17-yr-old female, subject 2 is a 56-yr-old female, subject 3 is a 65-yr-old female, and subject 4 is a 32-yr-old male. Immediately after lumbar puncture, the CSF was centrifuged at 800*g* for 5 min at 4°C to remove any cellular debris, and either directly used for analysis or stored frozen in small volumes at –80°C. As a measure for blood contamination, we examined the presence of hemoglobin protein peaks by MS. CSF samples used in this study lacked evidence of blood contamination. Aliquots of frozen CSF were thawed on ice immediately prior to use. For the current study, one experimental group used CSF immediately after lumbar puncture and the second experimental group used CSF that had been exposed to one freeze/thaw cycle. A third experimental group contained Protease Inhibitor Cocktail (Sigma) and phosphatase inhibitor cocktails (Calbiochem Phosphatase inhibitor Set II) added directly to the sample after thawing the sample on ice to evaluate proteome instability caused by proteolysis or dephosphorylation.

Study Design

Within each experimental group, two sample sets were prepared for analysis. Each sample set contained seven replicate tubes containing 20 μL of CSF for each of eight time points: 0, 2, 4, 6, 8, 12, 16, and 24 h. One sample set was incubated at 23°C for the designated time points. The second sample set was incubated at 4°C for the appropriate time points. The third experimental group containing protease and phosphatase inhibitors was incubated at 23°C. At each time point, all samples were prepared immediately for protein profiling on the strong anion exchange (Q10) ProteinChips (Ciphergen Biosystems, Inc., Palo Alto, CA) as follows. Ten microliters of urea buffer (9 *M* urea/2.5% CHAPS) were added to each sample, vortexed, and 170 μL of HEPES, pH 7.3 were added. The samples were then placed on a micromix shaker for 10 min. The Q10 ProteinChips were placed in a bioprocessor (Ciphergen Biosystems, Inc.) and first equilibrated with 100 m*M* HEPES pH 7.3 for 10 min and excess buffer removed from the spots. One hundred microliters of sample were then applied to each well and incubated for 30 min. Replicate samples were applied in random order to the chip arrays. The ProteinChips were washed five times in high-performance liquid chromatography-grade water. Excess water was removed and two applications of 1.5 μL of sinapinic acid in 50% acetonitrile/0.3% v/v trifluoroacetic acid was added to each spot and the arrays were air dried.

Mass Spectrometry

The Q10 arrays were then read in a Ciphergen PBS IIC Chip reader system containing an autoloader (Ciphergen Biosystems). Spectra were generated using a laser intensity range of 190 and a detector sensitivity range of 8 to 9 with a mass deflector setting of 1000 Da for the low-mass range (1–20 kDa). These settings were kept constant for all chips analyzed in an experiment. Two mass spectra were obtained for each sample: one for the mass range from 1 to 20 kDa, and a second for the mass range from 20 to 160 kDa. For each Q10 ProteinChip array, a standard CSF sample was loaded onto one spot to measure chip-to-chip variability. The coefficient of variance (COV) for four selected m/z signals was less than 30% across all chip arrays. External calibration of the spectrometer was performed using the "7-in-1" peptide mix from Ciphergen Biosystems (vasopressin [1084.247 Da], somatostatin [1637.903 Da], porcine dynorphin A [2147.5 Da], human

ACTH [2933.5 Da], bovine insulin B chain [3495.941 Da], human recombinant insulin [5807.633 Da], and hirudin [7033.614 Da]). Human SOD1 (15591.4 Da) was also added to this mix.

Data Analysis

Protein profile comparisons were made after normalization of each spectrogram to total ion current, and raw spectral data consisted of 18,400 mass peak values for each individual sample. All samples within each sample set were prepared and analyzed at the same time within the same experiment. Spectra were analyzed using Ciphergen ProteinChip software (v3.2.1). Statistical analysis of all mass peaks was performed using the nonparametric Mann-Whitney test on the maximal intensity of each peak. Peak labeling was performed using second-pass peak selection with a signal-to-noise ratio of 1.5. Significance threshold was set of $p < 0.05$.

Results

We first analyzed the SELDI-TOF-MS spectra of human CSF incubated at 23°C between 0 and 24 h. **Figure 1** represents typical spectra between 2.5 and 20 kDa. Low-molecular mass m/z peaks between 3 and 8 kDa exhibit alterations that are evident at early time points and are most prominent by 24 h (**Fig. 1**). Spectral peaks near 14 kDa also exhibit significant peak intensity value alterations by 4 h at 23°C. We next examined and compared the spectral pattern of an identical CSF sample incubated at either 23 or 4°C.

Figure 2 depicts typical SELDI-TOF-MS spectra of human CSF for each time point at both 23°C (left panels) and 4°C (right panels). For display purposes, we have magnified the spectral region between 11 and 15 kDa for representative spectra at each time point and temperature. As noted in the Methods section, we analyzed seven replicate samples for each time point and **Fig. 2** displays the representative spectra. Although small differences in average peak intensity levels are apparent for specific m/z peaks at various time points, we have highlighted one region of m/z peaks that exhibit significant variation over time at each temperature. The peaks between 13.7 and 14.1 kDa highlighted by "a" at 0 h resolve to a doublet (*) by 6 at 23°C.

At 4°C, the peaks between 13.7 and 14.1 kDa highlighted by "a" at 0 h resolve to a protein doublet (*) by 8–12 h at 4°C (**Fig. 2**, left panels). Finally, the spectrum observed after 6 h at 23°C is almost identical to the spectrum obtained after 16–24 h at 4°C (**Fig. 2**). Similar results were obtained for CSF samples that were exposed to one freeze/thaw cycle prior to analysis (data not shown). These data indicate that specific m/z peaks exhibit alterations over time regardless of the temperature but the stability of the CSF proteome is enhanced by maintaining samples at 4°C. The integrity of the mass spectral pattern may be maintained an additional 4–6 h by storing CSF samples at 4°C as opposed to 23°C.

We next determined if the addition of protease inhibitor and phosphatase inhibitor cocktails to the CSF would impede protein profile changes that occur over time. Spectra between 4 and 17 kDa for CSF incubated at 23°C between 0 and 8 h in the presence or absence of protease and phosphatase inhibitors are shown in **Fig. 3**. In the absence of any inhibitors, alterations in protein relative peak intensity values are evident within 2 h (**Fig. 3A**), similar to those described in **Figs. 1** and **2**. Immediately after addition of the cocktail inhibitors, many m/z peak intensity values exhibit decreased relative peak intensity, possibly as a result of the suppression of protein ionization from the protein chip surface (**Fig. 3B**). Other protein/peptide peaks near 7 kDa exhibit a transient increased relative peak intensity value. However, protein peak alterations are evident by 2 h even in the presence of protease and phosphatase inhibitors for peaks near 14 kDa, and the

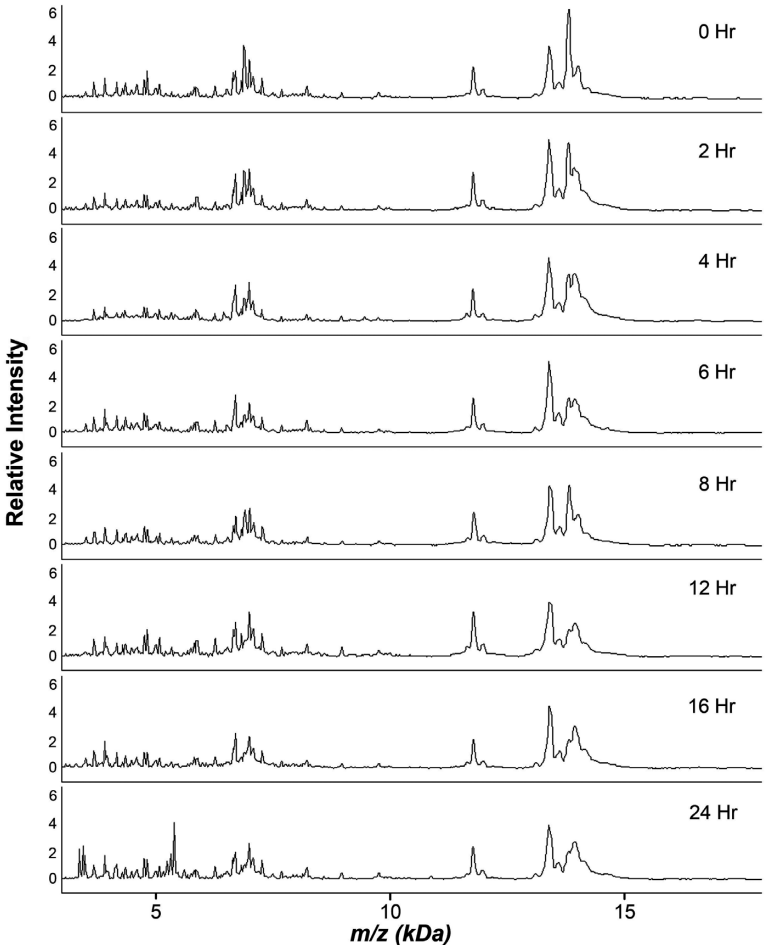

Fig. 1. SELDI-TOF-MS spectra of human CSF (subject 3, see Methods) incubated for various times at 23°C. The representative m/z profiles between 2.5 and 20.0 kDa are shown for 0, 2, 4, 6, 8, 12, 16, and 24 h.

spectral pattern for lower-molecular mass peaks is similar to that observed without protease and phosphatase inhibitors **(Fig. 3)**. The overall consistency of the spectral peak pattern between 0 and 8 h was higher for CSF lacking protease or phosphatase inhibitors. Similar results were observed using two different concentrations of inhibitors within the experiment. Therefore specific m/z peak changes that occur in CSF over time as detected by SELDI-TOF-MS are unlikely to be induced by protease or phosphatase activities.

To quantify proteomic changes over time, we first determined the average m/z peak intensity value and standard deviation using the seven replicate samples at each time point for the 23 and 4°C CSF samples. To characterize the trend in among-sample variability within each time point, we determined the variance (VAR) and the COV of each m/z position among profiles at each time point within each temperature data set. VAR is a measurement of the overall variation of the peak intensity values for a given m/z, and is computed as the average squared deviation for each m/z peak intensity value from the mean peak intensity value. VAR at 4°C increases slowly for the first 8 h, then plateaus and finally peaks

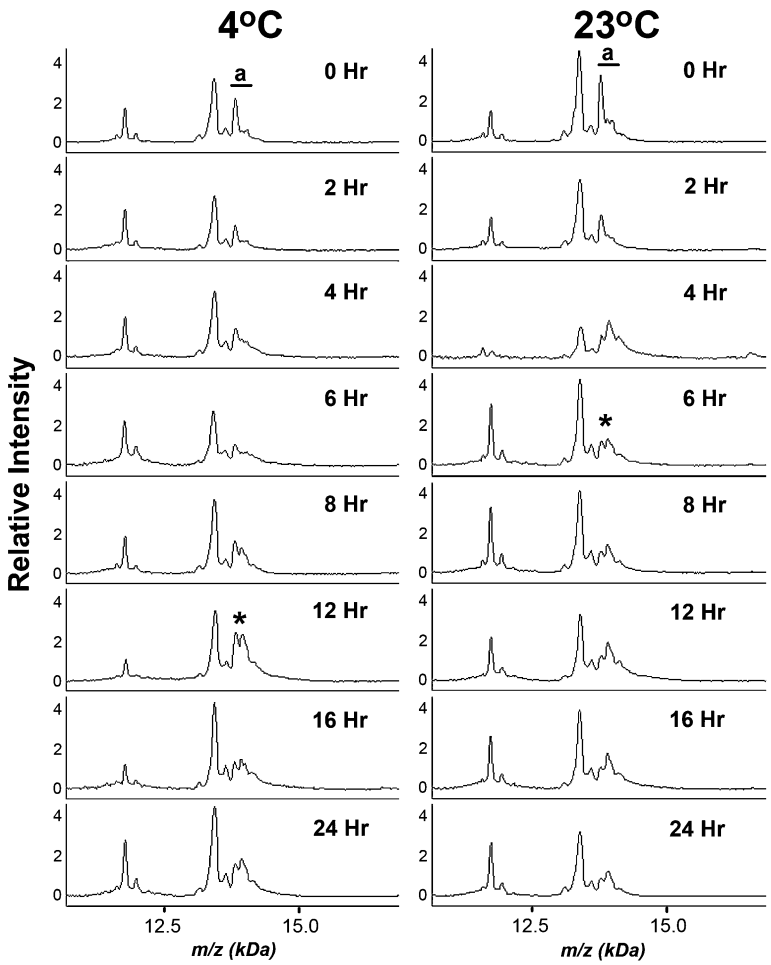

Fig. 2. SELDI-TOF-MS spectra from Q10 chips of human CSF (subject 4, see Methods) incubated for various times at either 4°C (**left panels**) or 23°C (**right panels**). The m/z profiles between 11.0 and 17.0 kDa are shown for 0, 2, 4, 6, 8, 12, 16, and 24 h. The arrow and region marked by "a" indicate m/z peaks exhibiting either alteration in relative peak intensity or peak resolution over time. * marks the presence of a protein peak doublet present at 6 h at 23°C that is apparent at 8–12 h at 4°C.

at 24 h **(Fig. 4A)**. This data suggest a high degree of reproducibility within spectra for individual samples at each time point. Even at 23°C, the variance at different time points remains low, again suggesting that any changes occurring within the proteome occur at a consistent rate within the replicate samples at each time point. COV at each m/z position was calculated as the standard deviation over the mean of each variance. Again, the COV measure exhibits no particular trend at either temperature, suggesting that reproducibility within each time point is quite good **(Fig. 4B)**. The observed small changes may reflect variation in baseline within the time points.

The COV for each time point were then used to calculate a whole-profile grand mean sum of mean deviation (GMSD) from the 0-h time point of each temperature. The GMSD was calculated as the sum of all mean of squared differences between each position in

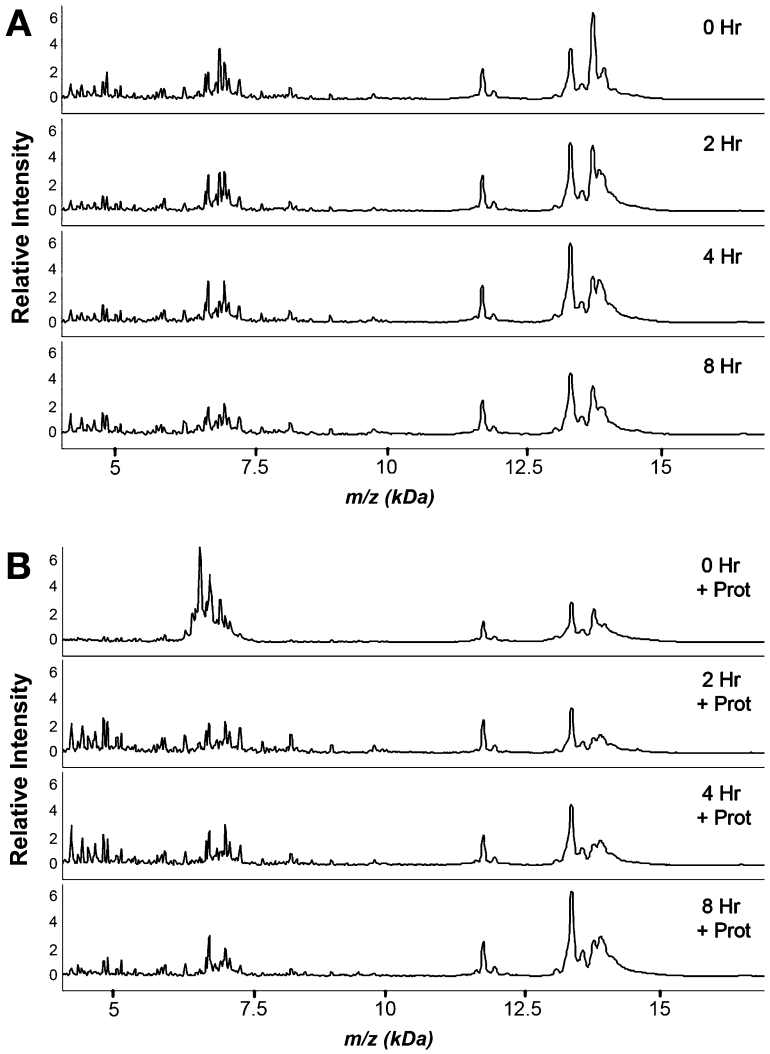

Fig. 3. Protease and phosphatase inhibitors fail to impede proteomic alterations over time at 23°C. CSF from subject 3 was incubated at 23°C for various lengths of time in the absence **(A)** or presence **(B)** of protease and phosphatase inhibitors. The m/z profiles between 11.0 and 15.0 kDa are shown for 0, 2, 4, and 8 h. **(A)** Proteomic alterations that occur over time at 23°C are similar to that observed in **Fig. 1**, even though the CSF is from a separate subject. **(B)** Addition of protease and phosphatase inhibitors results in immediate decreased peak intensity values for many m/z peaks at 0 h, although similar peak alterations occur at 4 and 8 h to CSF in the absence of inhibitors.

the mean profile at each time point and the mean profile at 0-h for each temperature (**Fig. 5**). GMSD from the 0-h time point exhibits an overall increasing trend at each temperature. For the 4°C samples, increased variability is initially observed at 8 h, suggesting proteomic alterations have occurred within 8 h at this temperature. For the 23°C samples, there are proteome alterations within 4 h as depicted by increased GMSD and then a large increase in GMSD occurs between 16 and 24 h (**Fig. 5**). The large increase in GMSD may reflect additional alterations of low-molecular mass peaks during this time period as evident

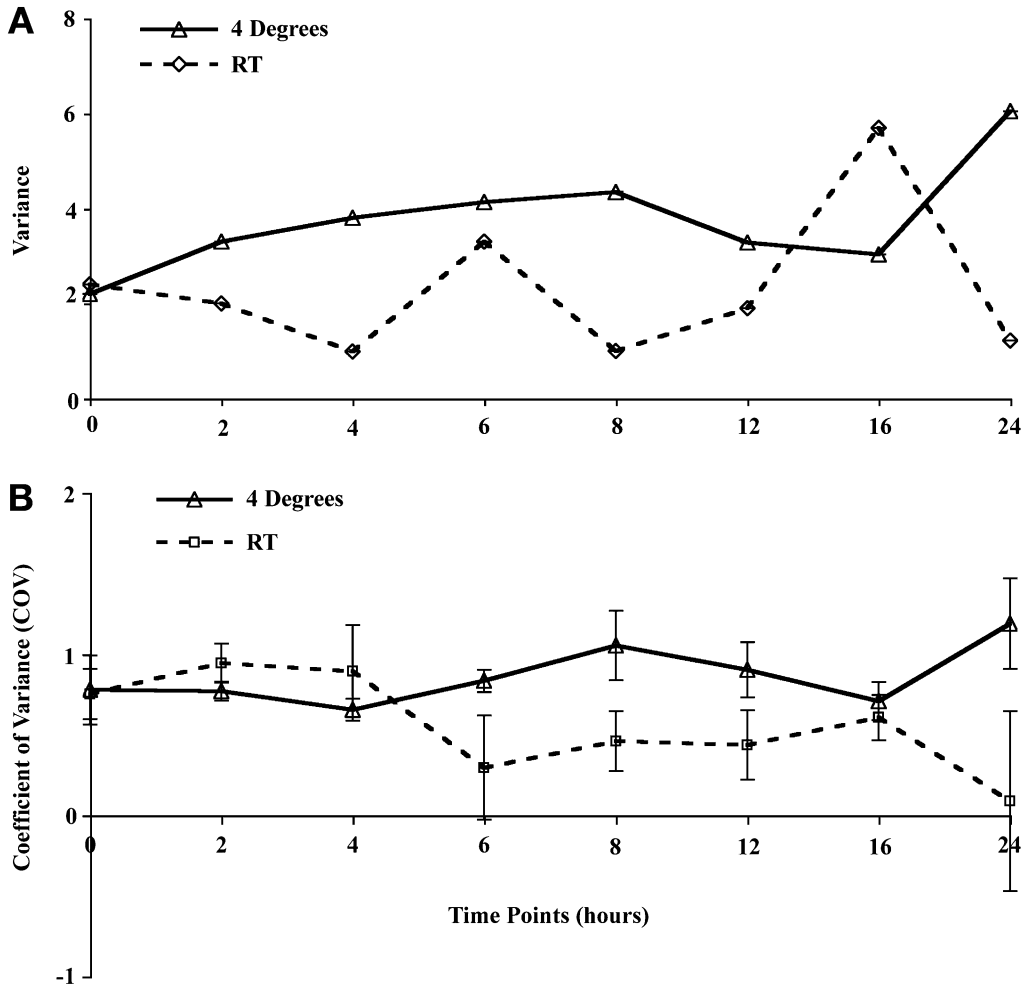

Fig. 4. **(A)** Variance of the m/z values within all replicate samples at each time point. For each time point, the average m/z peak intensity value and standard deviation was determined for each of the 18,400 mass peaks within the raw spectral data for all replicate samples. The average overall variance was then determined for each time point and represented as the percent variance with corresponding standard error of mean. **(B)** The coefficient of variance for each time point was then determined as the ratio of standard deviation over its mean for each time point. Dashed lines represent 23°C (room temperature) and solid lines 4°C samples in each panel. RT, room temperature.

in Fig. 1. These results suggest the proteome instability is more rapid and extensive at 23°C as compared to 4°C.

Discussion

The goal of this study was to define procedures that preserve proteomic stability of clinical samples and minimize artifacts that may arise when using MS-based proteomics to identify CSF-based biomarkers. Our results indicate that significant CSF proteomic alterations occur within 2–4 h at 23°C and within 6–8 h at 4°C. Further protein peak alterations were evident at later time points, most prominent in low-molecular weight species between 2 and 8 kDa. The addition of protease and

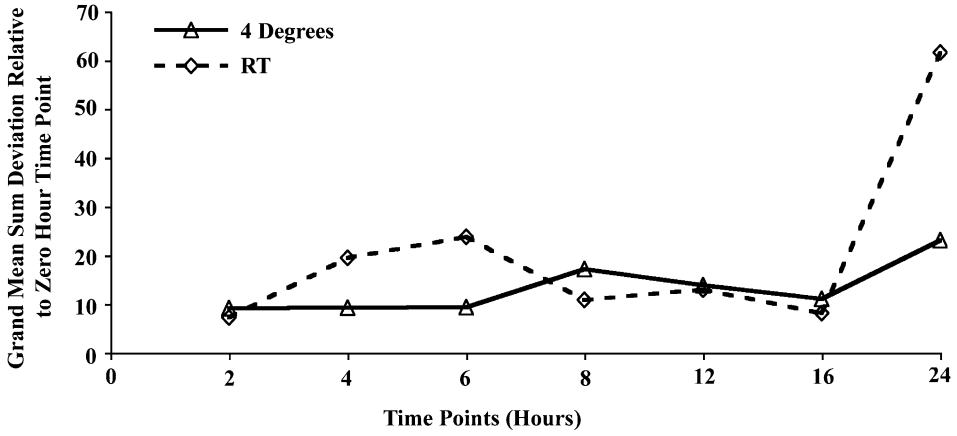

Fig. 5. Statistical comparison for the proteome stability at each time point to the 0-h time point. For both 23°C (room temperature [RT], dashed line) and 4°C (solid line) samples, the difference between the mean profiles at each time point relative to the 0-h time point mean profile was calculated as the grand mean sum deviation. This value, representing the cumulative departure from initial conditions, was plotted for each of the seven time points. At 23°C, changes in proteome stability when compared to the 0-h time point occur within 3 h and peak between 16 and 24 h. Proteome instability at 4°C is not apparent until the 8-h time point.

phosphatase inhibitor cocktails resulted in an immediate suppression of many spectral peaks, possibly because of suppression of protein ionization. Within 2 h, low-molecular mass peaks were now evident in CSF containing the inhibitor cocktails (**Fig. 3B**), although continued suppression of peak ionization was evident in many samples (data not shown). The presence of protease and phosphatase inhibitors failed to impede proteomic alterations significantly over time, although the presence of specific mass ion peaks was delayed by 2–4 h. Because the overall consistency of the mass spectral data, especially at the early time points between 0 and 8 h, was greater in samples lacking inhibitor cocktails, we suggest that rapid CSF recovery, preprocessing, and storage at –80°C will generate more stable mass spectral data than CSF with added protease and phosphatase inhibitors. However, the observed proteomic alterations may be induced by enzymatic activities not targeted by the inhibitor cocktails used in this study. Future studies will purify individual m/z peaks that change over time to determine their protein identity and potential mechanisms that induce the proteomic alterations. These findings are also important because it appears likely that proteomic biomarker patterns associated with disease, disease progression, and therapy responses may reflect biochemical interrelationships among peptides or proteins, which can be expected to change with temperature and time.

Therefore, care should be taken to monitor carefully the time necessary to harvest and process CSF samples until they are preferably snap-frozen and properly stored at –80°C.

Although many m/z peaks were quite stable even up to 24 h, variability within sample preparation and handling procedures may introduce significant protein alterations such that final mass spectral data analysis is difficult to properly interpret. Exposure of CSF samples to ambient temperatures should be minimized to maintain stability of the proteome within individual samples. Recent evidence suggests that ex vivo proteolytic processing of serum proteins results in peptide patterns characteristic for myocardial infarction ***(23)***. Our protocol

for rapid processing of CSF at 4°C provides one approach to reduce sample-to-sample proteome variability that may well result in the identification of "artifact" protein biomarkers.

Conclusion

Our data highlight the importance in identifying and following appropriate and consistent sample collection procedures when preparing clinical samples for proteomic analysis. CSF sample preprocessing time should be kept to less than 4 h and samples maintained at 4°C prior to long-term storage at –80°C to generate high-quality CSF samples for proteomic analysis.

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