

Proteomic Analysis of Cerebrospinal Fluid Changes Related to Postmortem Interval

ERIN J. FINEHOUT,¹ ZSOFIA FRANCK,¹ NORMAN RELKIN,² and KELVIN H. LEE^{1*}

Background: The study of proteins with altered production in postmortem cerebrospinal fluid (CSF) compared with antemortem CSF may improve the understanding of biochemical changes that occur immediately after death.

Methods: Two CSF samples (1 antemortem and 1 postmortem) were collected from 7 patients and analyzed by 2-dimensional gel electrophoresis. An analysis was also performed to identify proteins that showed a correlation between concentration change and postmortem interval. Tandem mass spectrometry was used to identify the proteins.

Results: Fifty-four protein spots were identified that showed a consistent and significant change in concentration in the postmortem CSF of all 7 patients (>3.5 -fold, $P < 0.01$). The proteins in these spots derive from a variety of functional groups, including cytoskeletal proteins, enzymes involved in glycolysis, and proteins that prevent oxidative stress. Fourteen protein spots were found to have an increase in production that correlated with postmortem interval.

Conclusions: Changes in protein production of postmortem vs antemortem CSF were studied. The proteins observed to change production in the postmortem CSF include several proteins previously observed as potential stroke biomarkers.

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Changes in brain biochemistry attributable to disease or injury can result in altered concentrations of individual proteins in the cerebrospinal fluid (CSF)³ in living persons (1). The protein content of CSF has also been found to change upon death (2, 3). For example, it is known that the concentration of several enzymes, including creatine

kinase B, increases within hours of death (3). Such postmortem changes in CSF protein may result from cell death, breakdown of the blood–brain barrier, or other events that are initiated when an individual dies. It has been proposed that a comparison of antemortem and postmortem CSF protein production may yield biomarkers for some neurological disorders (2). Accordingly, several studies used enzyme activity assays to measure postmortem changes in human CSF proteins such as lactate dehydrogenase, β -glucuronidase, and acid phosphatase (3, 4). In another study, postmortem ventricular CSF from 4 individuals was compared with the antemortem lumbar CSF from 4 other control participants by 2-dimensional gel electrophoresis (2DE) to separate and visualize the proteins (2). This proteomic approach identified 13 protein spots showing a significant increase in postmortem CSF compared with antemortem CSF. Almost all of these proteins were intracellular proteins, suggesting that they were released into the CSF upon cell death. In subsequent studies, those authors demonstrated that at least 2 of the proteins (nucleoside diphosphate kinase A and posttranslationally modified prostaglandin D-2 synthase) have an altered serum concentration after stroke (5, 6).

We used 2DE to compare antemortem and postmortem CSF. In contrast with previous studies, this analysis compares antemortem and postmortem CSF from the same individuals. This unique collection of antemortem and postmortem CSF from 7 individuals, together with the use of a fluorescent protein stain and laser scanning, enabled the identification of 54 protein spots that show a consistent change in production in postmortem CSF samples. The ability to compare antemortem and postmortem CSF from the same individuals decreases the chance that the observed changes are a direct or indirect result of genetic variability. To explore the question of whether there may be a relationship between the postmortem

¹ School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY; ² Department of Neurology and Neuroscience, Cornell University Medical College, New York, NY.

*Address correspondence to this author at: 120 Olin Hall, Ithaca, NY 14853. Fax 607-255-9166; e-mail: KHL9@cornell.edu.

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³ Nonstandard abbreviations: CSF, cerebrospinal fluid; 2DE, 2-dimensional gel electrophoresis; PMI, postmortem interval; %volume, percent integrated intensity; MS/MS, tandem mass spectrometry.

interval (PMI) and observed changes in protein amounts, perhaps caused by cell lysis or breakdown of the blood-brain barrier, a correlation analysis was performed for production changes and PMI.

Materials and Methods

SAMPLES

Both antemortem and postmortem CSF samples were collected from 7 individuals by the Institute for Brain Aging and Dementia at the University of California, Irvine, CA. The PMIs were 1.5–9.5 h. The antemortem samples were collected by lumbar puncture, and ventricular CSF samples were collected postmortem during autopsy. The samples were frozen at the brain bank and shipped to Cornell on dry ice. The samples remained frozen until used. The project has been reviewed and approved by the Cornell University committee on human participants (protocol 01-01-003).

On the basis of autopsy reports, 6 of the individuals were diagnosed with Alzheimer disease and the 7th individual with Pick disease. The Alzheimer patients (4 male, 2 female) were 62–88 years old (at death), and the Pick disease patient (female) was 44 years old. The mean disease duration was 9 years, and the causes of death, according to the autopsy, were either respiratory failure or unknown. CSF from unaffected persons was not used because of the difficulty in finding samples of both antemortem and postmortem CSF from a single healthy individual. It is possible that observations based on 7 individuals with dementia, 6 of whom have Alzheimer disease, would confound the analysis; however, CSF protein changes that have previously been reported for Alzheimer disease are typically less than the 3.5-fold cutoff used in this study (7, 8). Thus, we expected the changes observed to be a result of death rather than the patients' previous disease state.

2DE

The details of the protocols used for performing 2DE have been previously published (9). Briefly, 250 μ L of CSF (containing \sim 100 μ g of protein) were precipitated with ice-cold ethanol. The protein pellet was then dissolved in a solution of 9 mol/L urea (Bio-Rad Laboratories), 2% 2-mercaptoethanol (J.T. Baker), 2% IGEPAL (Sigma-Aldrich), and 0.25% carrier ampholytes (Bio-Rad). The protein mixture was then loaded onto an 18-cm, 3–10 nonlinear immobilized pH gradient isoelectric focusing gel (GE Healthcare). Isoelectric focusing was then performed at 20 °C with the Protean IEF unit (Bio-Rad) for a total of 100 kVh to separate proteins in the first dimension by isoelectric point. The immobilized pH gradient gels were equilibrated in solutions containing dithiothreitol (Bio-Rad) and, subsequently, iodoacetamide (Fluka) for reduction and alkylation of the focused proteins. In the second dimension, polyacrylamide gel electrophoresis was performed with 12%–15%T gradient slab gels to separate proteins by size. The gels were fixed, stained with SYPRO

Ruby Protein Gel Stain (Molecular Probes), and destained for 24 h in a solution of 10% methanol and 7% acetic acid. The gels were scanned on an FLA-3000 Fluorescent Image Analyzer (Fuji Photo Film Company).

GEL IMAGE ANALYSIS

The gel images were imported into a 2D gel analysis software package, Melanie v4.0 (GeneBio). Spots were autodetected by the software and the detected spots were manually edited to remove technical artifacts. A reference gel image was created by combining the spots present in 1 antemortem gel and 1 postmortem gel. The reference gel image contained all possible spots from these 2 gels. The protein spots from each of the 14 samples were then matched to this reference image to allow an intergel spot comparison. Spot changes that occurred in all individuals were included, and any spot changes that were not present in a particular individual were excluded from this study. Thus, any spots appearing in samples that were not used to create the reference image were not matched and not included in the subsequent analysis. Matching was initially performed with the automatic matching function in the Melanie software and was then manually edited by a single individual to correct for missed or incorrect matches. The percent integrated intensity (%volume) of each spot on each gel was then exported. The fold change for each spot was calculated by dividing the postmortem %volume by the antemortem %volume from the same patient.

Previous studies report a gradient in protein concentration between the ventricle and lumbar regions (10, 11). Individual protein concentrations have also been shown to change up to 3.5-fold between these 2 regions (12). Therefore, we used the convention suggested by Conti et al. (13) to compare protein concentrations between these regions: a change had to be at least 3.5-fold to be included in this study. The fold change data were analyzed to identify protein spots that demonstrated at least a 3.5-fold change in %volume for all 7 patients.

A correlation analysis was performed in the R environment v2.0.1 (14) with the difference in %volume (i.e., postmortem %volume – antemortem %volume) and the PMIs. The correlation analysis measures the correlation coefficient for each spot. The correlation coefficient, which can be from –1 to 1, is a measure of the strength of the relationship between the difference in %volume of that spot and the PMI. A positive coefficient indicates a positive relationship (i.e., as the PMI increases, the difference in %volume increases), and a negative coefficient indicates a negative relationship. The closer the absolute value of the coefficient is to 1, the greater the correlation between the 2 variables (15).

PROTEIN IDENTIFICATION

Some of the proteins in the 2DE spots of interest were identified with our 2DE CSF map on the basis of tandem mass spectrometry (MS/MS) identifications with $P < 0.05$

(16). The protein spots that were not previously identified in this antemortem CSF map were identified by tryptic digestion followed by MS/MS (4700 Proteomics Analyzer, Applied Biosystems) by previously published methods (17). Peptide mass fingerprint data were collected in positive reflector mode from 900 to 4000 m/z . An MS/MS analysis was performed on several of the highest intensity nontrypsin peaks. The spectra were analyzed with GPS Explorer (v3.0, Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot search engine (v1.97, (18)). The spectral data were submitted for a combined MS and MS/MS search against a local copy of the NCBI nr sequence database (19). A mass tolerance of 25 ppm was used for the MS data and a relative molecular mass tolerance of 0.2 for the MS/MS data. A value of $P < 0.05$ (calculated by GPS Explorer) was required for a protein identification to be considered correct.

Results

Examples of 2DE CSF gels from a study patient are shown in Fig. 1. The mean (SD) number of detected protein spots is 1233 (275) on the antemortem gels and 1462 (454) on the postmortem gels. A Student t -test analysis of the %volume data from all of the spots indicated that 310 protein spots showed a statistically significant ($P < 0.01$) change in production between the antemortem and postmortem samples.

An analysis of the data identified 54 protein spots with a fold change ≥ 3.5 for postmortem vs antemortem CSF in all 7 patients. These spots are numbered and labeled with a circle in Fig. 2A. The labeled protein spots covered a wide range of relative molecular masses and isoelectric

points. Significantly more spots had an increased %volume in the postmortem samples than had a decreased %volume (53 increased and 1 decreased). The spots with increased amounts ranged from those showing a mean 5-fold change to those detectable only in the postmortem samples. An example of 2 spots showing an increase in %volume in the postmortem CSF of a patient is shown in Fig. 2B. The proteins in many of the spots have been identified, and this information is summarized in Table 1.

The correlation analysis of the difference in %volume (postmortem minus antemortem) and PMI data identifies 284 protein spots with a correlation coefficient > 0.6 . When only the spots that showed a consistent increase in postmortem CSF across all patients were included, the list was decreased to 14 spots, which are identified in Table 2. This list contains several spots not in Table 1, because the spots did not show a fold change > 3.5 in samples with a smaller PMI. These spots are labeled with a triangle in Fig. 2A. Examples of the change in %volume as a function of PMI are shown in Fig. 3 for several protein spots.

Discussion

The fold change analysis revealed 54 protein spots demonstrating a consistent change in CSF concentration upon death. One may speculate that spot changes at lower relative molecular masses may derive from intact proteins of a higher relative molecular mass and that the change in amount is a result of proteolytic degradation upon death. The data in Table 1 demonstrate that the observed proteins cover a broad range of relative molecular masses (12 000–65 000), and the observed and expected relative molecular masses based on an intact protein are similar for most of the entries. The majority of the proteins in

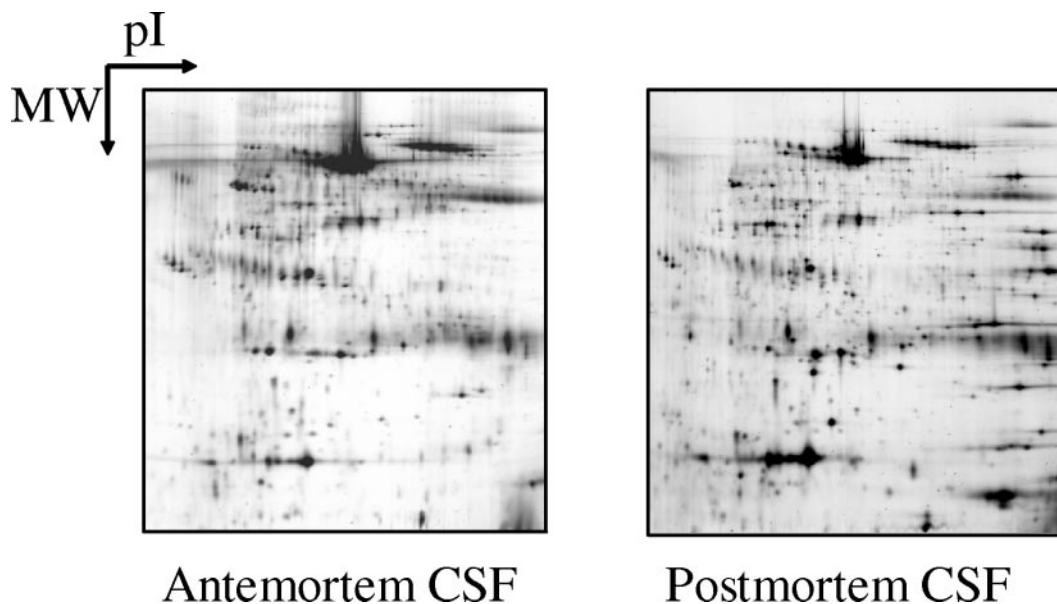


Fig. 1. Examples of antemortem and postmortem CSF separated by 2DE. Both CSF samples are from the same patient.

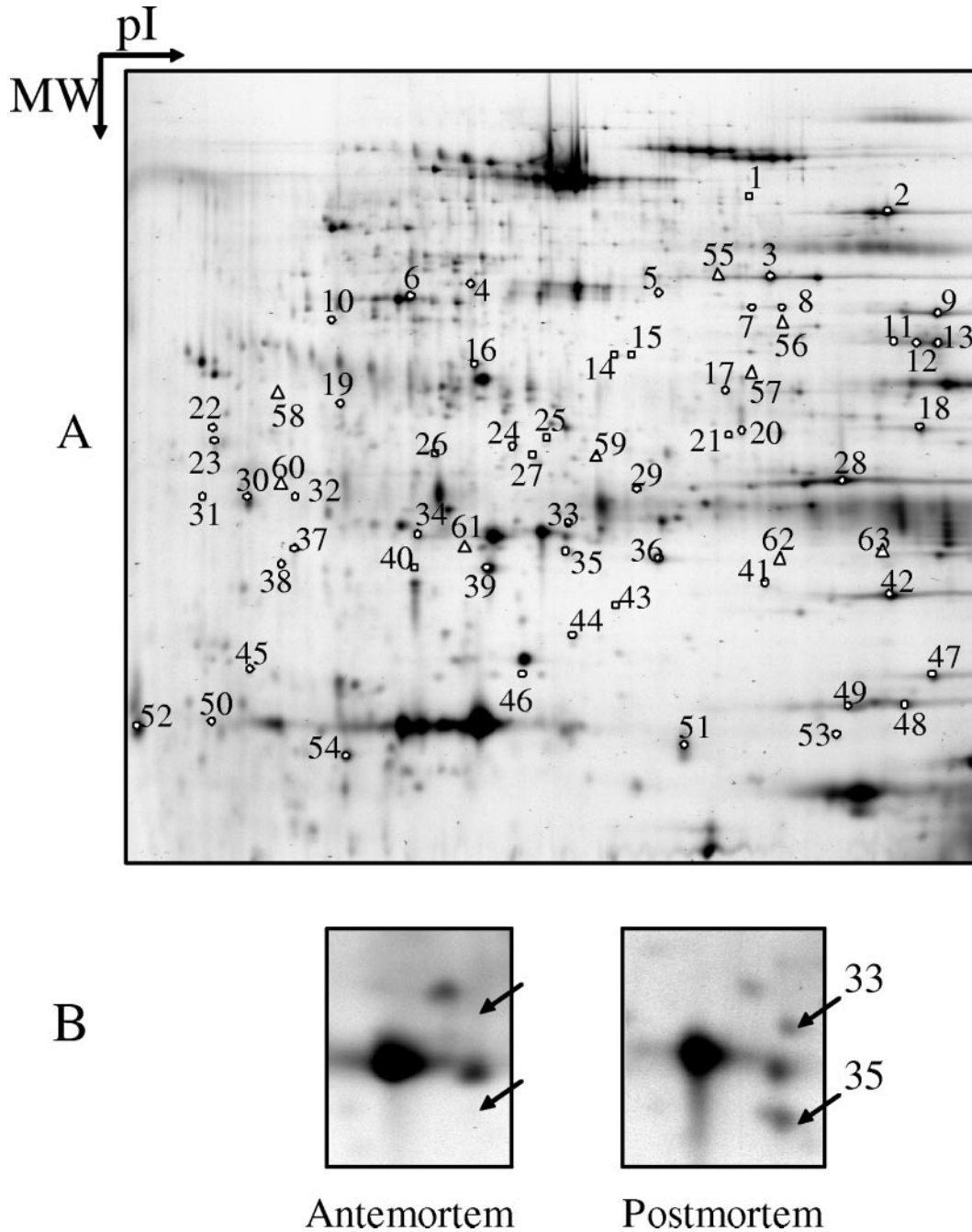


Fig. 2. (A), 2DE gel from a postmortem CSF sample showing the location of protein spots listed in Tables 1 and 2.

Circles mark the protein spots that show a consistent and significant change in %volume between the ante- and postmortem samples. The circles are labeled 1 through 54. Triangles are used to mark the protein spots that show a correlation between %volume and PMI but did not show a significant change in %volume at all PMIs. The triangles are labeled 55–63. (B), an example of protein spots with an increased %volume in the postmortem CSF sample for a patient. The spot numbers correspond to those in 2A.

Table 1 are intracellular proteins that are not expected in CSF, which is consistent with their release into the CSF upon cell death.

Several of the observations about proteins in Table 1 are consistent with previously published findings from postmortem CSF. For example, postmortem CSF has been reported to have an increase in the concentration of creatine kinase B (3). In the present study, the mean

%volume of creatine kinase B was found to increase 12-fold in postmortem CSF compared with antemortem CSF from the same individual. The results of the current study also included several of the proteins that Lescuyer et al. (2) observed to increase in postmortem CSF compared with antemortem CSF in antemortem and postmortem CSF samples that were not obtained from the same cohort. These confirmed proteins include cathepsin D,

Table 1. Proteins identified in spots showing a consistent-fold change >3.5 when comparing postmortem and antemortem CSF.^a

Spot no.	Protein ID	NCBI nr accession no.	Δ	Observed M_r (in thousands)	Calculated M_r (in thousands)	Mascot score
1	Stress induced phosphoprotein 1	5803181	+	65	63	346
2	Pyruvate kinase	478822	+	63	58	346
3	α enolase	29792061	+	50	47	494
4	Creatine kinase B	49457530	+	49	43	74
5	No ID		+	48		
6	Actin β	14250401	+	46	42	439
7	No ID		+	44		
8	NADP-dependent isocitrate dehydrogenase	3641398	+	44	47	230
9	Phosphoglycerate kinase	48145549	+	44	45	362
10	No ID		+	43		
11	No ID		+	40		
12	Aldolase A	4930291	+	40	39	466
13	Aldolase A	4930291	+	40	39	486
14	α -Enolase	29792061	+	39	47	163
15	No ID		+	39		
16	No ID		+	37		
17	Malate dehydrogenase	7431153	+	35	36	101
18	Carbonyl reductase 1	4502599	+	32	30	465
19	No ID		+	34		
20	Esterase D	33413400	+	32	31	101
21	Pyrophosphate phospho-hydrolase	33636766	+	32	29	72
22	No ID		+	32		
23	Calbindin 1	4826655	+	30	30	220
24	CAPZ B protein	19352984	+	30	21	107
25	No ID		+	31		
26	Cathepsin D, chain H	5822091	+	29	26	90
27	Cathepsin D, chain H	5822091	+	28	26	181
28	Carbonic anhydrase II	809316	+	27	29	503
29	No ID		+	25		
30	Proteasome subunit, α type, 5	53734656	+	25	26	96
31	No ID		+	25		
32	No ID		+	25		
33	NRH: quinone oxidoreductase 2 B-chain	5822494	+	24	26	151
34	TPA: LRRC15	34146792	+	23	64	113
35	No ID		+	22		
36	Oncogene DJ1	31543380	+	22	20	274
37	No ID		+	22		
38	No ID		+	21		
39	Peroxiredoxin 2	33188452	+	21	16	432
40	Peroxiredoxin 2	2507619	+			— ^b
41	Neuropolypeptide h3	4261934	+	21	16	37i
42	Neuropolypeptide h3	4261934	+	20	16	332
43	Tetranectin	4507557	–			— ^b
44	Nucleoside-diphosphate kinase 1	38045913	+	18	20	230
45	No ID		+	17		
46	Stathmin 1	51895905	+	17	17	31i ^c
47	Cofilin (nonmuscle)	30582531	+	16	19	206
48	Peptidylprolyl isomerase A	51895760	+	15	18	192
49	Peroxiredoxin 5, chain H	16975162	+	15	17	232
50	Transthyretin	339685	+	14	13	36i
51	Fatty acid binding protein 5	30583737	+	13	15	132
52	Calmodulin 3	58218968	+	14	17	146
53	No ID		+	13		
54	No ID		+	12		

^a The spot number corresponds to the label in Fig. 2A. The direction of change (Δ) is shown as is the approximate observed M_r , the calculated M_r , the Mascot MOWSE score, and the NCBI nr accession number (19). A positive Δ indicates the expression of the protein was greater in the postmortem CSF. Protein identifications are based on a combined MS and MS/MS search except where noted. ID, identification.

^b Identification made using previously published 2DE CSF map (16).

^c i indicates that score is based only on MS/MS data.

Table 2. Spots identified to have a change in %volume that correlated with the PMI.^a

Spot no.	Protein ID	NCBI nr accession no.	Correlation coefficient
3	α -Enolase	29792061	0.80
17	Malate dehydrogenase	7431153	0.74
40	Peroxiredoxin 2	33188452	0.90
47	Cofilin (nonmuscle)	30582531	0.85
53	No ID		0.67
55	α -Enolase	29792061	0.62
56	No ID		0.62
57	Glyoxylate reductase	6912396	0.92
58	No ID		0.85
59	Haloacid dehalogenase-like hydrolase domain	14149777	0.69
60	No ID		0.70
61	No ID		0.60
62	Proteasome subunit, β type 2	30583113	0.73
63	No ID		0.79

^a The spot number corresponds to the labels in Fig. 2A. ID, identification.

nucleoside diphosphate kinase 1, fatty acid binding protein 5, oncogene DJ1, and peroxiredoxin 5.

Table 1 also includes proteins that have not been previously identified in the context of antemortem vs postmortem changes in CSF proteins. Several of these proteins, including stathmin 1, neuropolypeptide h3, and TPA:LRRC15, are known to be highly produced in the brain (20). Some of the proteins in Table 1 are involved in

the glycolysis/carbohydrate metabolism pathways, and several other proteins are believed to be prevent oxidative stress. These 2 categories of proteins are altered after stroke-induced damage to the brain and after death.

Phosphoglycerate kinase, aldolase, pyruvate kinase, NADP-dependent isocitrate dehydrogenase, α -enolase, and malate dehydrogenase are involved in glycolysis or carbohydrate metabolism and are observed to be in-

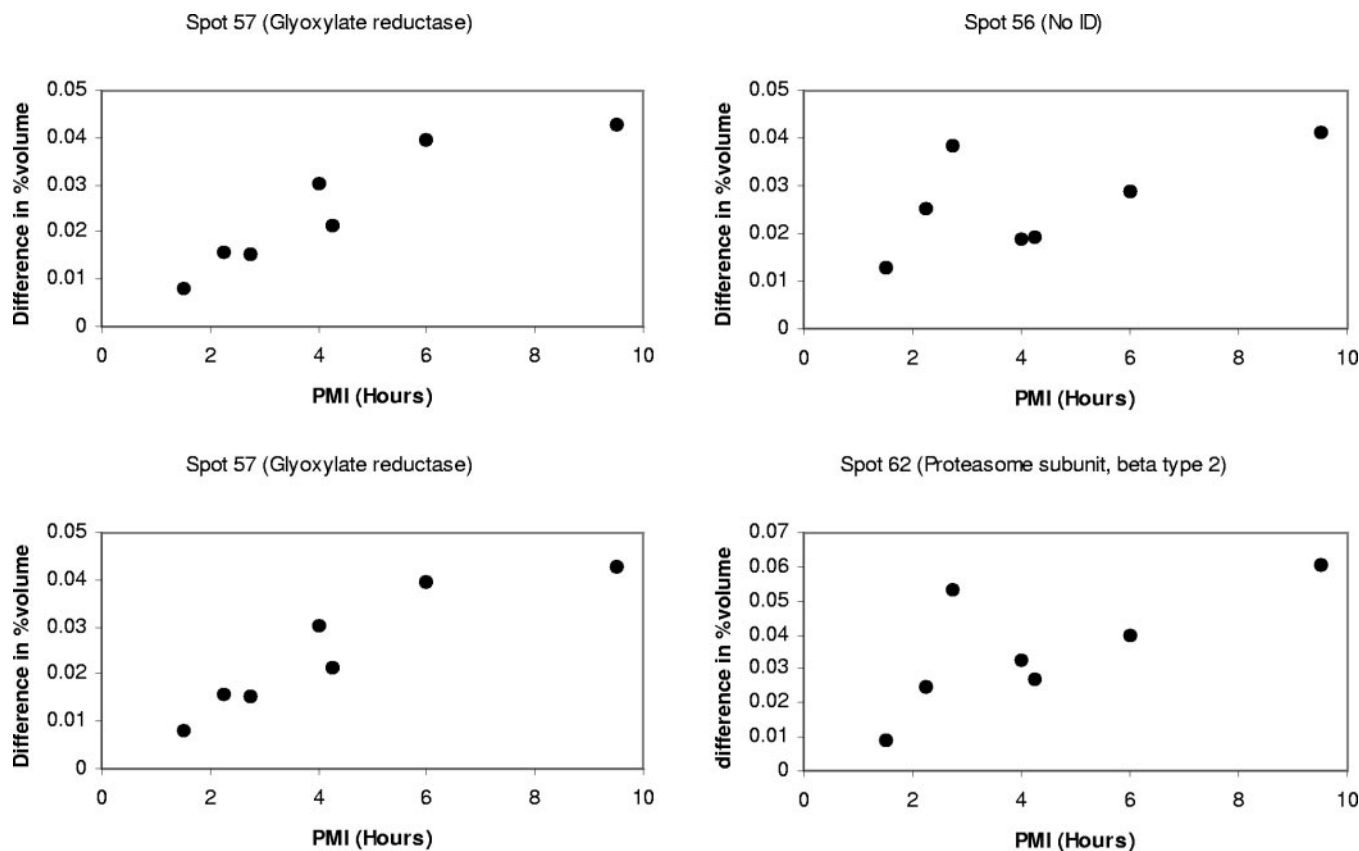


Fig. 3. Examples of the change in %volume vs PMI for several of the protein spots found to have a correlation score >0.6.

creased upon death. Previous investigations of the brain microdialysate have found that the concentration of glucose decreased and that of lactate increased with an occurrence of hypoxia/ischemia (21, 22). This observation is consistent with a change in energy metabolism in an injured brain, and postmortem changes in CSF proteins have been proposed as a model for studying brain injury (2). Experiments with cultured rat astrocytes have also demonstrated that hypoxia induces cells to increase their glycolytic capacity, leading to an increase in the concentration of enzymes such as pyruvate kinase (23).

Oxidative stress is also known to play a major role in the pathogenesis of brain injury (24). Some evidence exists that after cerebral ischemia, oxidative stress is a primary mediator of neurologic injury (25). Several of the identified proteins (peroxiredoxin 2, peroxiredoxin 5, carbonyl reductase 1, and quinone oxidoreductase 2) have been shown to play a role in protecting cells from oxidative stress (26–28). All of these proteins show an increase in the postmortem CSF sample compared with the antemortem samples.

The 2 other kinases identified in this study, creatine kinase B and nucleoside-diphosphate kinase 1, have previously been found to increase in concentration after stroke, and differences in CSF proteins from antemortem and postmortem samples have been previously proposed as a means to identify stroke biomarkers (2). Increases in nucleoside-diphosphate kinase 1 have been reported in blood (5), and increases in creatine kinase B have been reported in both blood (29) and CSF (30, 31).

The proteins in Table 1 that do not derive from these previously discussed categories include cytoskeletal proteins (actin β , CAPZ β subunit, cofilin, and stathmin 1), transport-related proteins (calbindin 1, fatty acid binding protein 5, transthyretin, and tetranectin), proteolytic proteins (cathepsin D and proteasome subunit α type 5), and proteins with other or unknown functions (stress-induced phosphoprotein 1, peptidylprolyl isomerase A, oncogene DJ1, TPA:LRRC15, neuropolypeptide h3, esterase D, pyrophosphate phosphorhydrolase, carbonic anhydrase, and calmodulin 3).

In terms of correlating changes with PMI, previous studies have found that the CSF amounts of some enzymes increase with increasing PMI and hypothesize that this is because of progressive cellular breakdown (3). The 14 spots that showed a consistent increase in the postmortem CSF and a correlation score >0.6 are shown in Table 2. This table contains proteins from most of the previously discussed functional groups, and no one group is represented significantly more than the others. Among these, previous work demonstrates a correlation between increased CSF concentration of α -enolase and the volume of an infarction (32).

By performing a proteomic analysis on antemortem and postmortem CSF samples from 7 individuals, we identified several proteins that demonstrate altered CSF amounts upon death. A correlation analysis indicates that

some of these CSF proteins have amounts that change with PMI. Taken together, these observations of the CSF protein changes may provide a better understanding of changes that occur upon death and that relate to PMI. Furthermore, it has been proposed that such changes may lead to validated biomarkers for stroke. However, additional studies of a larger cohort, ideally with antemortem and postmortem CSF samples from stroke patients, are necessary to confirm these observations in that context.

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