Proteomic Assessment of Important Proteins for Motor Recovery in a Rat Model of Photochemically-Induced Thrombosis

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ABSTRACT

Using a rat model of focal photochemically-induced thrombosis, we demonstrated previously the presence of activated areas in the ipsilateral sensorimotor cortex surrounding areas of cerebral infarction. Using the same model, we next used the images of two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) and peptide mass fingerprinting (PMF) analysis to screen key proteins upregulated in the activated area. 2D-PAGE and PMF identified Laminin receptor 1, Apolipoprotein-E, Enolase 2, Contactin, DnaJ and Glial fibrillary acidic protein delta as the main proteins. The results suggest that astrocytes, which seem to be related to these proteins, play an important role in behavioral recovery in this model.

INTRODUCTION

Stroke is the third leading cause of death in most developed countries, including Japan, after cancer and heart disease. It is also a major cause of morbidity, long-term disability, hospital admission, and healthcare costs1. We have used the rat model of brain infarction with photochemically-induced thrombosis (PIT) caused by platelet aggregation [2,3]. The technique used in this model is simple and allows researchers to induce a focal and uniform brain lesion. The hindlimb hemiparesis induced by unilateral motor cortex lesion shows complete recovery at 10 days with least variability among rats after infarction[4,5]. By allowing us to develop permanent cortical lesions relevant to clinical settings, this rat model permits comparison of the effects of experimental treatments with sufficient sensitivity to detect minor differences between treatments through detailed evaluation of post-stroke behavioral recovery. Using the PIT model and functional magnetic resonance imaging (fMRI), we reported previously the activation of an area in the contralateral non-injured sensorimotor cortex, in addition to the ipsilateral sensorimotor cortex of infarction[6]. A similar phenomenon was observed in humans using fMRI[7,8]. A recent study reported that the human brain exhibits adaptive functional changes after focal damage[9]. Using the PIT model, we next used kynurenic acid treatment to counteract key molecules identified by the Gene Chip and observed differential rates of behavioral recovery after infarction[10]. The results also indicated the potential importance of the ipsilateral cortex surrounding the lesion in motor recovery

after brain damage in this model[11].

The ipsilateral cortex surrounding cortical lesion contributes to functional neuron-reorganization of the neural networks and modulates behavioral recovery after PIT. The present study is an extension to our previous research and was designed to evaluate the ipsilateral cortex surrounding the lesion by analyzing the key proteins involved in PIT by 2-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) and by the peptide mass fingerprinting (PMF) analysis. A better understanding of the molecular mechanisms of post-stroke axonal sprouting may provide a framework for the development of therapies to improve functional recovery.

MATERIALS AND METHODS

Animals

The experiments described in the present study were carried out in accordance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" of the Physiological Society of Japan. In addition, this study was carried out in compliance with the institutional guidelines of animal experiments at The Jikei University School of Medicine. A total of 24 male Fischer rats, aged 7 weeks, were used in this study. All rats were kept under standardized conditions (12–12 h light-dark cycle, lights on 7 a.m. and off 7 p.m., with free access to food and water), which were the same conditions used in our previous studies (4-6, 10). Rats were separated into three groups for 1) 48 h after injury (n = 8), 2) 96 h after injury (n = 8) and 3) control (n=8).

Photochemically-induced thrombosis

Rats were anesthetized with isoflurane (induction 3.0% in air; maintenance 1.5% in air) and placed in a stereotaxic frame (Narishige, Tokyo, Japan). After skin incision and cutting the galea aponeurotica, an 8-mm diameter light beam, with a 150 W cold-type halogen light (PICL-NEX, Japan), was focused on the skull and centered over the right hindlimb sensorimotor cortex at bregma –1.0 mm, 2.0 mm lateral to midline12. An aluminum foil tape was placed on the left side of the skull parallel to midline to avoid damage to the left-side brain. The right femoral vein was exposed and cannulated using a needle attached to a microinjection pump. Rose Bengal, 80 mg/kg (dissolved in 0.9% saline), was infused at a rate of 60 μl/min. Light exposure commenced 90 sec after the end of Rose Bengal infusion and lasted for 15 min. Sham controlled rats were anesthetized and injected with Rose Bengal solution, the skin and galea were cut, but the brain was not exposed to the light beam over the following 15-min period. Thus, the only difference between intervention and control rats was exposure to light. The rats were sacrificed at 48 or 96 hours after infarction. A rectangular block measuring 2 mm in the sagittal direction x 5 mm in the coronal direction x 3 mm deep was obtained from the right sensorimotor cortex excluding the infarct lesion. Similarly, same-size rectangular blocks were obtained from the right sensorimotor cortex of control rats.

2D-PAGE

Then, 5-fold volume of T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) was applied, containing Protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following application of 0.15 g zirconium beads (2 mm), the extraction mixture was homogenized (4,000 rpm for 60 sec, 4) in Beads Homogenizer (TOMY, Micro Smash MS-100R). The homogenate was centrifuged (20,000 x g for 20 min) and the supernatant was used for determination of protein concentration by the Bradford method (Bradford reagent, Sigma). Protein concentrations were approximated by using a standard curve of BSA. This protein extraction solution (200 mg protein) was precipitated in -30°C cold acetone for 3 hr and the precipitate was collected by centrifugation (20,000 x g for 15 min). Acetone was discarded and the precipitate was allowed to dry up for a few minutes at room temperature. The protein precipitate was treated with 250 ml of IEF lysis buffer and vortexed

gently for 45 min at room temperature. The IEF lysis buffer contained 6 M urea, 2 M thiourea, 3% CHAPS, 1% Triton X-100, and DeStreak reagent (Amersham Biosciences, Arlington Heights, IL). After lysis of proteins in IEF lysis buffer, the solution was centrifuged (20,000 x g for 20 min). The IEF sample solution was rehydrated in Immpbiline DryStrip gel (Amersham Bioscience, pH 3-10 non-linear, 13 cm) for 12 hr. After rehydration, IEF was performed at 150 V for 1 hr, 5,000 V ramping for 2.5 hr, and 5,000V IEF for 15 hr. With regard to 2D-PAGE conditions, before 2D-PAGE, the strip gel was equilibrated in sample buffer (strip equilibration buffer; 6 M urea, 20% glycerol, 2% dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), and 375 mM Tris-HCl, pH 8.8) for 45 min. 2D-PAGE was performed at 25 mA CC for about 4 hr (10-18 %, 14 x 14 cm polyacrylamide gradient gel). SDS-PAGEed gel was stained overnight with 0.02% CBB G-250 staining solution. After staining, the gel was destained in 5% acetic acid. Images were acquired with GS-800 calibrated Imaging Densitometer (Bio-Rad, Hercules, CA).

Peptide Mass fingerprinting in gel digestion

The gel pieces were washed with water while vortexing and dehydrated with acetonitrile. Acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. Next, 10 mM dithiothreitol in 100 mM ammonium bicarbonate was added and the solution was incubated for 1 hr at 56°C. The solution was replaced by the same volume of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and incubated for 45 min at room temperature in the dark with vortexing. The gel pieces were washed with 100 mM ammonium bicarbonate while vortexing, dehydrated with acetonitrile and rehydrated again with 100 mM ammonium bicarbonate and dehydrated again. Acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. Trypsin solution was added and the solution was incubated for 45 min at 4°C. Trypsin solution contained 50 mM ammonium bicarbonate, 5 mM calcium chloride, and 10 μg/ml Etrypsin. After incubation at 4°C, the solution was re-incubated for 12 h at 37°C. Peptides were extracted using sequential steps of 20 mM ammonium bicarbonate, followed by 5% formic acid in 50% acetonitrile. The extracted peptides were dried in a vacuum centrifuge. Then, 0.1% trifluoroacetic acid was added and were desalted using ZipTip C18 (Millipore, Bedford, MA) according to the protocol provided by the manufacturer. The peptides were eluted with 0.1% trifluoroacetic acid in 50% acetonitrile and applied to the MALDI plate.

Mass spectrometry

Mass spectrometry of peptides was performed using MALDI-TOF MS AXIMA-CFR (Shimadzu Corporation).

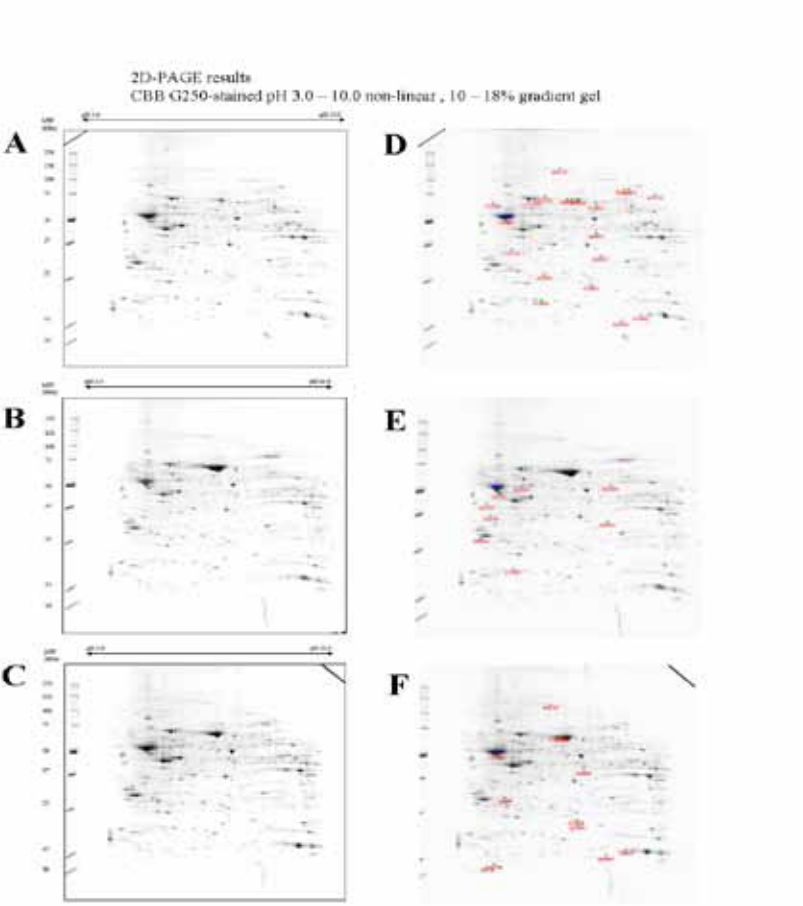
Database analysis

Proteins were identified using Mascot search (Matrix Science, http://www.matrixscience.com/).

Immunohistochemistry

Two animals of each group were perfused transcardially with heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was dissected out and postfixed overnight. Brain coronal sections were then washed in 0.1 M phosphate-buffered saline (PBS) and incubated with 0.1 M PBS containing 10% normal donkey serum and 1% Triton X-100 for 1 h at room temperature (RT) to block nonspecific staining. For immunodetection of glial fibrillary acidic protein (GFAP), the sections were incubated with the primary antibody diluted with 0.1 M PBS containing 5% normal donkey serum and 0.3% Triton X-100 for 1 h at RT. The sections were washed in 0.1 M PBS and incubated with the secondary antibodies diluted with 0.1 M PBS containing 5% normal donkey serum, and 0.1% Triton X-100 for 1 h at RT. We used a mouse anti-GFAP antibody (1:500; Millipore) as the primary antibody and a rhodamine-conjugated donkey anti-mouse IgG as the secondary antibody (Jackson Immunoresearch Laboratories, West Grove,

Fig. 1. Images of 2D-PAGE (CBB G250-stained pH 3.0-10.0 non-linear, 10-18% gradient gel). A: Control (n=8), B: 48h after injury (n = 8), C: 96h after injury (n = 8).



D: 2D-PAGE image on A (with marked 20 proteins that were expression more than three times between control and 48h after PIT). E: 2D-PAGE image on B (with marked 8 proteins that were expressed more than three times between 48h after PIT and 96h after PIT). F: 2D-PAGE image on C (with marked 12 proteins whose expression increased more than three times from control to 96h after PIT).

Fig. 2. *Immunodetection of GFAP in the ipsilateral cortex surrounding the lesion at bregma –1.0 mm. A and B: Control, C and D: 48h after injury, E and F: 96h after injury. *Lesion site.*

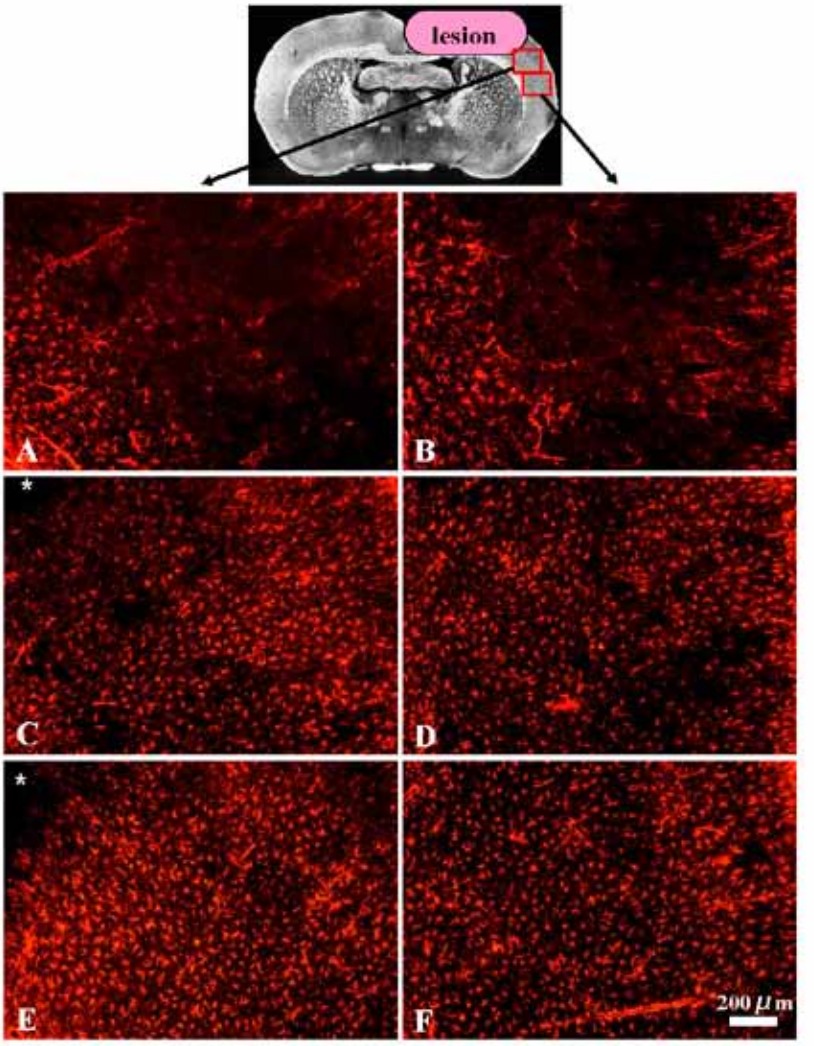


Table1. *Mascot Search Results. Summary of eight identifiable proteins that showed significant differences in expression levels and hit_score.*

spot #	accession	hit_ mass	pI value	hit_ score	title_text	Norm Qty		
						Cont	48h	96h
SSP0403	gi|8393693	32918	4.8	138	laminin receptor 1 (*Rattus norvegicus*)	168.8	78.7	301.2
SSP1301	gi|1703338	35788	5.23	166	Apolipoprotein E precursor (Apo-E)	250.4	154.0	645.0
SSP1513	gi|26023949	47510	5.03	221	enolase 2, gamma (*Rattus norvegicus*)	1781.4	430.8	2161.7
SSP2605	gi|5030428	48809	5.72	428	glial fibrillary acidic protein delta (*Rattus norvegicus*)	10.3	185.5	1256.3
SSP6608	Mixture	0		268	Mixture of proteins: "gil109499524","gil1711535"	795.3	254.8	811.7
SSP4901	gi|1363280	114301	5.8	226	contactin1/F3 - rat	132.6	838.3	638.2
SSP5106	gi|84000579	20793	5.99	175	ferritin light chain 1 (*Rattus norvegicus*)	52.2	243.3	665.5
SSP5406	gi|62543491	40755	5.92	79	DnaJ (Hsp40) homolog, subfamily B, member 11 (*Rattus norvegicus*)	54.2	211.8	228.5

PA). The sections were then washed in 0.1 M PBS, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence-stained sections were viewed on an Olympus microscope with epifluorescence. Images were acquired with a personal computer coupled with CCD camera (Coolsnap cf, Roper).

RESULTS

A total of 2,139 spots were identified in the three groups. Figure 1 shows images of 2D-PAGE for the control rats (A), rats sacrificed at 48h after PIT (B), and 96 h after PIT (C). Figure 1D is a 2D-PAGE image with marked 20 proteins whose expression increased more than three time from the control to 48h after PIT, while Figure 1E is an image of 8 proteins whose expression increased more than three times between 48h and 96h after PIT, and Figure 1F is an image of 12 proteins whose expression increased more than three times between control and 96h after PIT. A total 40 spots were expressed more than three times. Comparison between control and 48h after PIT and between control and 96h after PIT showed 8 spots were identical. These 8 spots were not recognized in other comparisons. Therefore, mass spectrometry was performed on 32 spots. Only eight spots showed certainty (hit score >75). The results are shown in Table 1. These proteins were divided three patterns.

PIT resulted in reduced expression of Laminin receptor 1, Apolipoprotein E precursor (Apo-E) and Neuron specific gamma Enolase at 48h followed by significant increase at 96h after PIT. PIT also resulted in increases in the expression of Glial fibrillary acidic protein (GFAP) and Ferritin light chain 1 at 48h and 96h after PIT. Expression of DnaJ (Hsp40) and Contactin1/F3 increased from the control to 48h after PIT.

Figure 2 shows immunodetection of GFAP. A higher expression of GFAP was observed in the cortex surrounding the lesion side after PIT. Furthermore, GFAP expression was higher at 96h compared to at 48 h after PIT.

DISCUSSION

Hemiparesis of the hindlimb induced by unilateral motor cortical lesion shows complete recovery at 10 days with least inter-individual variability among rats after PIT4,5,10,11. In the present study, rats were sacrificed at 48 hours and 96 hours after PIT. We used the cortex surrounding the lesion, which is considered to participate in recovery of paralysis, and compared this area with the same area of control rats. In this model, hemiparesis of the hindlimb shows clear improvement between 2 and 3 days, 5 days and 6 days after infarction, based on previous studies[4,5,11]. Thus, we considered that gene expression and certain key proteins should appear before improvement of hemiparesis, when animals were sacrificed at 48 and 96 hours after PIT.

Improvement of hemiparesis after brain injury is nearly always accompanied by brain network changes[13], and sprouting[14]. Stroke induces a process of axonal sprouting in peri-infarct tissue that results in a substantial re-mapping of the connections of the somatosensory cortex adjacent to the infarct[14,15]. Injury of the central nervous system (CNS) induces expression of both growth-promoting and growth-inhibitory genes that together determine the location and degree of axonal sprouting[14]. In fact, we demonstrated previously the upregulation of mRNAs of three genes (glycine receptor, α1-subunit, fibroblast growth factor receptor 4, insulin-like growth factor 1) in the target area10. Furthermore, we also confirmed the functional importance of altered expressions of these receptors in the recovery process in the PIT model, using kynurenic acid as an antagonist for glycine/glutamate receptors[10]. Insulin-like growth factor 1 showed increased protein expression in activated astrocytes in the ischemic penumbra[16].

The present study showed that the proteins involved in the functional recovery were Laminin receptor, Apo-E, Neuron specific gamma Enolase, GFAP, Ferritin light chain, DnaJ (Hsp40) and Contactin1/F3. Four of these proteins are involved in

various astrocyte functions. Laminins, which are large multidomain glycoproteins of the extracellular matrix, are the major non-collagenous constituents of the basement membrane[17]. They are implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and enhancement of the metastatic phenotype of tumor cells[17,18].

In neurocytes, Apo E expression correlates with the promotion of nerve protective action, and regeneration of injured neurocytes. In the CNS, astrocytes mainly produce and secrete Apo E[19]. Through the lipid transportation function, they maintain dendritic connections at the synapse and restoration of neurocytes. Contactin is a member of the immunoglobulin superfamily. It is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a neuronal cell adhesion molecule implicated in axogenesis and myelination[20]. Contribution of astrocytes to activity-dependent structural plasticity in the adult brain[20].

GFAP, an intermediate filament protein of mature astrocytes in the CNS, plays specific roles in astrocyte functions[21]. Phosphorylation of GFAP regulates the structural plasticity of glial filaments and eventually the functions of astrocytes[21]. The higher expression of GFAP in the cortex surrounding the lesion side reflects enhanced astrogliosis. Likewise, PMF analysis indicated increased expression from 48 to 96 h after PTI. Considering that functional neural reorganization of the network at this area contributes to behavioral recovery, it is possible that astrocytes play a positive role in neurite outgrowth and nerve protection.

Expression of Neuron specific gamma Enolase, Ferritin light chain and DnaJ (Hsp40) seems to be influenced by PIT-induced brain damage. DnaJ is a heat shock protein and functions in association with DnaK (Hsp70). DnaJ plays a key role in chaperone reactions by stimulating the ATPase activity and activating the substrate binding of Hsp70. Manipulation of the cellular stress response offers strategies to protect brain cells from damage caused by ischemia and neurodegenerative diseases[23]. Overexpression of Hsp70 reduced ischemic injury in the mammalian brain[23].

Ferritin is a ubiquitous and highly conserved protein, which plays a major role in iron homeostasis by sequestering and storing iron in a non-toxic and soluble form. Since ferritin is an acute-phase reactant, its level is often elevated in infection[24]. Neuron specific gamma enolase encodes one of the three enolase isoenzymes found in mammals. This isoenzyme, a homodimer, is found in mature neurons and cells of neuronal origin. A switch from alpha enolase to gamma enolase occurs in neural tissue during development in rats and primates [25]. Neuron specific gamma enolase also increase significantly during the first week of ischemic stroke[26].

Recent reports have demonstrated that the adult mammalian brain has the capacity to regenerate some neurons after various insults[27,28]. Subventricular zone (SVZ) GFAP-expressing cells labeled by a cell-type-specific viral infection method were found to generate neuroblasts that migrated toward the injured striatum after middle cerebral artery occlusion. These neuroblasts in the striatum formed elongated chain-like cell aggregates similar to those in the normal SVZ, and these chains were closely associated with thin astrocytic processes and blood vessels28. Further studies should be conducted to evaluate the relationship between generation of neuroblasts and progress of recovery in our PIT models.

In conclusion, we showed that Laminin, Apo-E, Enolase 2, contactin, ferritin light chain, DnaJ and GFAP are important proteins involved in recovery from hemiplegia in the rat thrombosis model. In particular, the results showed that astrocytes, which are related to these proteins, play an important role in behavioral recovery in the PIT model.

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