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A Study of Glutathione *S*-transferase pi Expression in Central Nervous System of Subjects with Amyotrophic Lateral Sclerosis Using RNA Extraction from Formalin-Fixed, Paraffin-Embedded Material

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The expression of glutathione *S*-transferase pi (GST pi), an enzyme responsible for inactivation of a large variety of toxic compounds was studied in spinal cord, motor and sensory brain cortex obtained from patients who died in the course of amyotrophic lateral sclerosis (ALS). The studies were performed on formalin-fixed, paraffin-embedded (FFPE) and freshly frozen tissues. The method of RNA isolation from FFPE was modified. A significant decrease of GST pi-mRNA expression was found in cervical spinal cord and motor brain cortex of ALS subjects comparing to analogue control tissues ($P < 0.01$), as well as in motor cortex of ALS subjects comparing to their sensory cortex ($P < 0.05$). In spinal cords the decrease in GST pi-mRNA expression was accompanied by a decrease of GST pi protein level. Results indicated lowered GST pi expression on both mRNA and protein levels in the regions of nervous system affected by ALS. The non-properly inactivated by GST toxic electrophiles and organic peroxides may thus contribute to motor neurons damage.

KEY WORDS: Amyotrophic lateral sclerosis; brain cortex; formalin-fixed; Glutathione *S*-transferase pi; paraffin-embedded material; spinal cord.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive degeneration and loss of motor neurons. The disease is familial in 5–10% of cases, the one fifth of which are linked to mutations in SOD gene (1). In about 95% of all cases ALS is a sporadic disorder of unknown origin. Its susceptibility determining factors underlying the neuronal loss remain unclear. The toxicity of environmental factors, excitotoxicity, neurotrophic factors deprivation and oxidative stress, have been suspected to participate in the neuron degeneration (2,3). Glutathione *S*-transferases (GST, EC 2.5.1.18) are a family of enzymes widely distributed in cells. Some of GST isoenzymes express both transferase and selenium-independent peroxidase activity (4,5). They can therefore protect cells against the toxicity of xenobiotics and contribute to antioxidant defense (6). Among the compounds inactivated by glutathione *S*-transferase there are toxic electrophiles such as aliphatic and aromatic heterocyclic

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radicals, epoxides and arene oxides, polycyclic aromatic hydrocarbons and isothiocyanates as well as harmful products of unsaturated fatty acids peroxidation, toxic quinone metabolites of catecholamines and drugs (7–10). Conjugation of those compounds with reduced glutathione catalyzed by GST leads to formation of less toxic and more hydrophilic, thus easier excreted products. GSTs are dimeric enzymes encoded by polymorphic family of genes (11). In humans, eight gene families of GST have been characterized for isoforms pi, mu, alpha, theta, zeta, sigma, kappa and chi (12–14). GST pi, mu and alpha are present in human central nervous system (15,16). The main isoform present in human brain is GST pi (15). It is encoded by one pi (P1) gene family localized on chromosome 11 (14). In the studies conducted on blood and cerebrospinal fluid of ALS patients we found that glutathione *S*-transferase activity and GST pi expression were decreased in peripheral blood mononuclear cells, and that GST peroxidase activity was raised in both CSF and blood serum (in press).

The aim of the present study was to establish the GST pi expression in spinal cord and both motor and sensory brain cortex of ALS subjects. Since it is very difficult to obtain samples of fresh tissue from autopsy of patients with ALS, we developed a technique which let us conduct GST pi-mRNA expression studies in formalin-fixed, paraffin-embedded material.

EXPERIMENTAL PROCEDURE

Chemicals. TRIzol® Reagent, dNTP, ribonuclease inhibitor, M-MLV reverse transcriptase, Taq polymerase were from Invitrogen, proteinase K was from ICN, oligo$(dT)_{15}$ primer was from Promega. Reagents for polyacrylamide gel electrophoresis were from Bio-Rad, rabbit anti-human GST-pi polyclonal antibody was from Novocastra, standard GST pi from human placenta from Sigma, ECL plus Western Blotting Detection System from Amersham.

All other chemicals were of the highest grade commercially available.

Material. The samples of formalin-fixed, paraffin-embedded (FFPE) cervical spinal cord, sensory and motor brain cortex were obtained from the archives of the Department of Neurology, Medical University of Warsaw. The tissues obtained from autopsy were fixed in neutral-buffered formalin and embedded in paraffin using standard procedures. Two groups were studied: the experimental, which comprised 15 patients (age 47–75 years, mean 64.3 $\pm$ 8.7) who died in the course of clinically and morphologically definite ALS, and the control, which comprised 10 patients (age 30–84 years, mean 62.5 $\pm$ 17.5) without any neurodegenerative disease. Interval between the patients death and tissues fixation ranged from 11 to 40 h. In ALS subjects the time from the disease onset till death ranged from 1 to 4 years.

The samples of fresh cervical spinal cord were obtained from autopsy of 2 ALS patients (age 63 and 66 years), and of 10 control cases without any neurodegenerative disease (age from 42 to 87 years, mean 59.3 $\pm$ 15.5 years). Autopsy was performed 1–3 days after death.

RNA Extraction. Paraffin from FFPE samples (20–50 mg) was removed by serial washings in xylene (4–8 times) and absolute ethanol (3–4 times). The deparaffinized samples were dried, homogenized in denaturation solution according to Lehmann and Kreipe (17) and incubated at 55°C overnight (16–18 h). The next day the samples were centrifuged (14,000 $\times$ *g*, 5 min at 4°C). From this point the Lehmann and Kreipe method (17) was modified and obtained supernatant (200 µl) was mixed with 1 ml of Trizol® Reagent instead with sodium acetete–phenol–chloroform solution. RNA was further isolated according to manufacturer's protocol. Trizol® Reagent was also used for RNA isolation from freshly frozen tissues.

cDNA Synthesis and PCR Amplification. The expression level of mRNA for GST pi was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Samples for RT-PCR in a volume of 20 µl contained: 2 µg of total RNA, 0.25 µg oligo$(dT)_{15}$ primer, 4 µl 5 $\times$ first-strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 0.2 mM of each dNTP, 10 mM DTT, 40 U ribonuclease inhibitor, 200 U M-MLV reverse transcriptase and diethyl pyrocarbonate-treated water. After incubation at 37°C for 60 min, the samples were heated in 94°C for 5 min and cDNA was stored at −20°C. Two microliters of cDNA sample was used as a substrate for PCR reaction in a 25-µl volume with 1 µM of forward and reverse primers, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5 µl 10 $\times$ PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl) and 1 U Taq polymerase. Specific oligonucleotide primers for GST pi analysis in FFPE samples were as follows: CCAGGTGACGCAG-GATGGTA (forward), GGCTCACTCAAAGCCTCCTG (reverse). The primers for beta 2-microglobulin (housekeeping gene, internal control) were: AAGATGAGTATGCCTGCCGT (forward), GATGCTGCTTACATGTCTCG (reverse). PCR reaction was carried out as follows: initial denaturation (3 min at 94°C) followed by 43 cycles (denaturation - 1 min at 94°C, annealing - 1 min at 60°C for GST pi and 55°C for beta 2-microglobulin), extension for 2 min at 72°C), and final extension step (7 min at 72°C).

Specific oligonucleotide primers for GST pi analysis in freshly frozen samples were used according to Marie et al.(18), and were as follows: GGCTCACTCAAAGCCTCCTG (forward), AGTGCCTTCACATAGTCATC (reverse). The specific mRNA sequence for beta 2-microglobulin was amplified as described by Brophy et al. (19) using following primers: CCAGCAGA-GAATGGAAAGTC (forward), GATGCTGCTTACATGTCTCG (reverse). The conditions of PCR reaction for freshly frozen tissues were identical as for FFPE samples except the number of cycles which were 28 for GST pi and 26 for beta 2-microglobulin.

Quantification of PCR Products. PCR products were separated on a 1.5% agarose gel with ethidium bromide (0.5 µg/µl). The level of specific mRNA was measured and expressed in semi-quantitative way as the ratio of optical density band of GST pi to optical density band of beta 2-microglobulin. The assay was repeated 2 times for each sample and performed in duplicate.

Western Blotting. Samples of freshly frozen spinal cord were homogenized in isotonic PBS, centrifuged (15 min, 12 000 $\times$ *g*, 4°C), and obtained supernatant was used for Western blotting. Western blotting was performed after electrophoresis in 14% polyacrylamide gel according to Laemmli (20), with rabbit anti-human GST-pi polyclonal antibodies. GST pi from human

placenta was used as a standard. Blots were visualized using ECL plus Western Blotting Detection System.

System UVI-KS4000, Syngen Biotech. was used for densitometric analysis of RT-PCR and Western blotting results.

Statistical Analysis. Results were expressed as means ± SD. Quantitative comparison between studied groups was performed by Student's t-test using Statistica software (StatSoft 6.1). Significance was set at $P < 0.01$ or 0.05 level.

RESULTS

The GST pi-mRNA expression in FFPE cervical spinal cords of 15 ALS subjects ranged from 0.79 to 1.09, and from 0.98 to 2.02 in controls. The mean value of GST pi expression in FFPE spinal cords was significantly lower in ALS (0.90 ± 0.14) than in controls (1.34 ± 0.41, $P < 0.01$) (Table I). In two freshly frozen spinal cords obtained from autopsy of ALS subjects, the GST pi-mRNA level was 0.76 and 0.64, and it was also lower than in freshly frozen control cervical spinal cords (mean 0.82 ± 0.12, ranged from 0.63 to 1.07, $n = 10$) (Table I).

Table I. Mean Expression of GST pi-mRNA in Central Nervous System

Tissue	Expression level		Significance
	ALS	Control	
FFPE spinal cord	0.90 ± 0.14	1.34 ± 0.41	$P < 0.01$
Fresh spinal cord	0.70 ± 0.05	0.82 ± 0.12	NC*
FFPE motor cortex	0.98 ± 0.26	1.39 ± 0.35	$P < 0.01$
FFPE sensory cortex	1.29 ± 0.31	1.38 ± 0.26	No

Data are the mean ± SD; *NC – not calculated.

The level of GST pi-mRNA expression in FFPE motor and sensory brain cortex ($n = 12$), obtained from the same control cases, was similar and accounted for 1.39 ± 0.35 and 1.38 ± 0.26, respectively. The mean GST pi expression in FFPE motor cortex of ALS subjects was 0.98 ± 0.26 ($P < 0.01$), and it was significantly lower than in motor cortex of the control cases. It was also lower than in sensory cortex of the same ALS subjects, where the mean value accounted for 1.29 ± 0.31 ($P < 0.05$) (Table I). The level of GST pi-mRNA expression in ALS sensory brain cortex was slightly lower than in control sensory cortex but the decrease was not significant (Table I). GST pi expression was decreased in motor cortex of all 12 ALS brains when compared to the expression in the sensory cortex (from the same brains) (Fig. 1), whereas in control brains the level of GST pi-mRNA in motor cortex was decreased in 5 out of 10 studied brains (Fig. 2).

GST pi expression on protein level was also reduced in freshly frozen samples of ALS spinal cord than in control spinal cords (Fig. 3).

DISCUSSION

It is very difficult to obtain fresh tissue samples from ALS patients right after autopsy. The previous studies of Rupp and Locker (21) have demonstrated

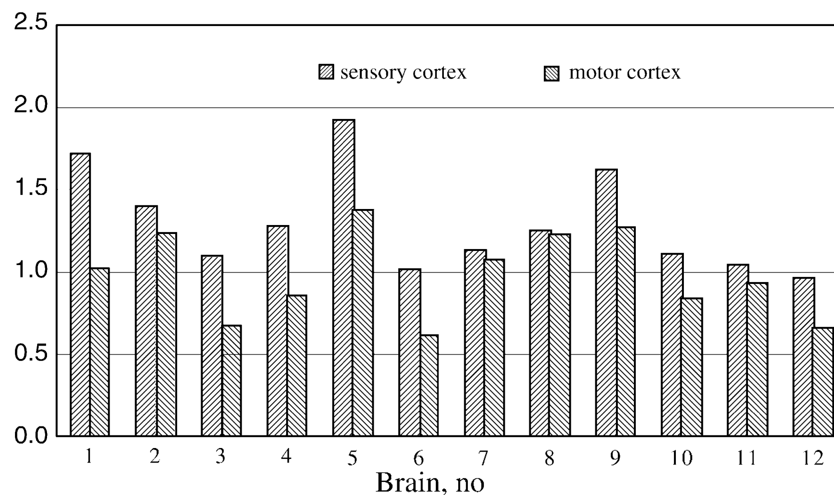

Fig. 1. GST pi-mRNA expression in brain cortex of individual ALS cases. RNA was isolated from FFPE sensory and motor brain cortex. RT-PCR was performed as indicated in Experimental Procedure section. Each value represents the mean of two determinations, each performed in duplicate.

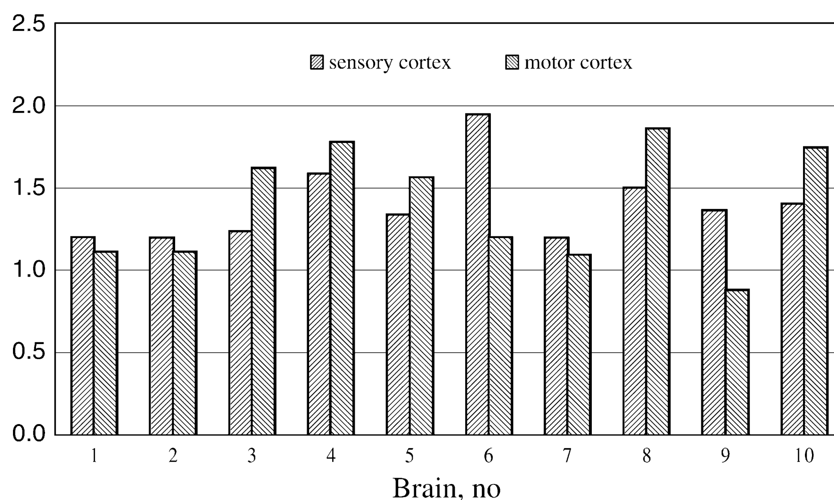

Fig. 2. GST pi-mRNA expression in brain cortex of individual control cases. RNA was isolated from FFPE sensory and motor brain cortex. RT-PCR was performed as described in Experimental Procedure section. Each value represents the mean of two determinations, each performed in duplicate.

that RNA extracted from formalin-fixed, paraffin-embedded material is suitable for gene expression determination. However, RNA isolated from FFPE tissues can be of poor quality because its extensive degradation might occur before completion of the formalin fixation processes. Moreover, formalin fixation causes cross-linkage between nucleic acids and proteins, and it covalently modifies RNA making subsequent RNA extraction, reverse transcription and quantitative analysis difficult (22).

In our preliminary study we tried to isolate RNA from FFPE specimens of spinal cord and brain cortex following the protocols described by various authors (17, 23, 24). Despite multiple attempts, we were not able to obtain enough RNA for PCR until we finally modified the original method of Lehmann and Kreipe (17) by replacing the extraction with sodium acetate-phenol-chloroform solution by the extraction with Trizol® Reagent. To confirm the results obtained in FFPE specimens, we also isolated RNA from freshly frozen spinal cords obtained straight from autopsy. Since the fixation of tissues in formaldehyde leads to extensive crosslinking and subsequent nucleic acids fragmentation (23), for the studies with FFPE specimens we designed PCR primers that resulted in much shorter PCR products than in case of freshly frozen tissues (97 bp for GST pi, 85 bp for beta 2-microglobulin, and 245 bp for GST pi, 268 bp for beta 2-microglobulin, respectively).

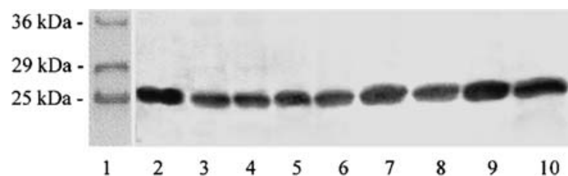

Fig. 3. Expression of GST pi protein in spinal cord. Expression was determined by Western blotting method as indicated in Experimental Procedure section. Comparable amounts of protein (6 μg) isolated from freshly frozen cervical spinal cords were run in each lane. Lane 1 – ladder, lane 2 – standard GST pi (100 ng), lanes 3, 4 – GST pi from ALS patient no 1, lanes 5, 6 – GST pi from ALS patient no 2, lanes from 7 to10 – GST pi from 4 control cases.

In both FEPE and fresh spinal cords the GST pi expression on mRNA level was lower than in control spinal cords. We were only able to study fresh spinal cords obtained from 2 ALS subjects. The results were however supported by data from Western blotting, which indicated decreased level of GST pi protein in both fresh spinal cords of ALS cases.

In motor brain cortex the GST pi-mRNA expression was significantly lower compared to the expression in sensory cortex of the same ALS subjects. It was also significantly lower than in motor cortex of control brains. The decrease in GST pi expression in spinal cord and brain motor cortex indicate that the process of detoxification catalyzed by GST is less efficient in ALS cases than in subjects without neurodegenerative disease. It is also less efficient in brain regions affected by amyotrophic lateral sclerosis compared to regions non-affected by the disease (e.g. sensory cortex). Consequently, the

exposure of these regions to toxic electrophiles and reactive organic peroxides may be higher in comparison to other parts of cns. However, more studies are needed to answer the question whether the decrease of GST pi expression observed in tissues affected in ALS is a specific cause of degeneration or if it occurs as a consequence of neurodegeneration.

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