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Functional characteristic of PC12 cells with reduced microsomal glutathione transferase 1*

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Microsomal glutathione transferase 1 (MGST1) possesses glutathione transferase and peroxidase activities and is active in biotransformation of xenobiotics and in defense against oxidative stress. To assess MGST1 role in the development and functioning of PC12 cells, we constructed a cell line with reduced MGST1 (PC12_M). Real-time PCR and immunoblot assays showed MGST1 expression lowered to 60% and immunocytochemical analyses demonstrated an altered concentration and distribution of the enzyme. PC12_M cells revealed a larger tendency to grow in clusters, weaker adhesion, irregular shape of bodies, short neurite outgrowth and higher percentage of necrotic cells (34%). The total GSTs activity determined with non-specific substrate CDNB (1-chloro-2,4-dinitrobenzene) decreased by 15–20%, whereas that with DCNB (2,4-dichloro-1-nitrobenzene), a substrate more specific for cytosolic GSTs, was similar to the one in control cells. This suggests that reduction of MGST1 cannot be compensated by other glutathione transferases. In PC12_M cells the total glutathione content was higher by 15–20%, whereas the GSSG/GSH ratio was lower than in control cells. Moreover, the laminin-dependent migration rate was much faster in control cells than in PC12_M, suggesting some alterations in the metastatic potential of the line with suppressed MGST1. The amount of MAP kinases (p38, JNK, ERK1/2) was elevated in PC12_M cells but their phosphorylation level declined. Microarray analysis showed changed expression of several genes, which may be linked with differentiation and necrosis of PC12_M cells. Our data suggest that MGST1 could be an important regulator of PC12 cells development and might have significant effects on cell growth and proliferation, probably through altered expression of genes with different biological function.

Keywords: microsomal glutathione transferase 1, PC12 cells; pseudoneuronal phenotype; necrosis; metastatic potential; signaling pathways

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INTRODUCTION

MGST1 (microsomal glutathione transferase 1) is a member of the MAPEG superfamily (membrane-associated proteins in eicosanoid and glutathione metabolism), which also includes LTC4 (leukotriene C_4) synthase, FLAP (5-lipoxygenase-activating protein), MGST2 and MGST3 (microsomal glutathione transferase 2 and 3) and MGST1-L1 (microsomal glutathione transferase 1-like 1) (Jakobbson *et al.*, 2000). Northern blot analysis showed that in rats the highest level of mRNA MGST1 was detected in the liver. Taking the amount in the liver as 100%, the MGST1 expression levels in some other tissues were: adrenal gland 13%, uterus 11%, stomach 8%, kidneys 6%, testis 5% and small intestine 3.8% (Estonius *et al.*, 1999). High amounts of MGST1 are detected in the endoplasmic reticulum, in the outer mitochondrial and plasma membranes of hepatocytes what is related to a specific role of this cell type. However, even lower presence of the enzyme in extra-hepatic tissues appears to be physiologically significant (Siritantikorn *et al.*, 2007).

MGST1 is a homotrimeric protein with a molecular mass of 51.9 kDa. Each subunit contains a single cysteine (Cys49), which is modified by many substrates, which increases the activity of the enzyme. MGST1 has been shown to be activated by reactive oxygen (O_2^-, H_2O_2) and nitrogen ($ONOO^-$, NO) species, sulfhydryl reagents, proteolysis, heating, radiation and dimerization of the homotrimers (Imaizumi *et al.*, 2006; Yanbin *et al.*, 2006). The enzyme has glutathione transferase and

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Abbreviations: Ache, acetylcholinesterase; Bad, BCL2-associated agonist of cell death; Bax, BCL2-associated X protein; BCL2, apoptosis regulator Bcl-2; Bcl-w, apoptosis regulator BCL-W; Bcl-x, BCL2-like 1; Bmp2, bone morphogenetic protein 2; Cdk5, cyclin dependent kinase 5; Chrm1, cholinergic receptor, muscarinic 1; CDNB, 1-chloro-2,4-dinitrobenzene; DAPI, 4,6-diamidine-2-phenylindole; DCNB, 2,4-dichloro-1-nitrobenzene; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); Efna 1, ephrin 1; ERK1 and ERK2, extracellular signal-regulated kinase 1 and 2; Fmr1, fragile mental retardation 1; Gpi, glucose-6-phosphate isomerase; GSH, reduced glutathione; GSSG, glutathione disulfide; GSTs, glutathione transferases; GSX, total glutathione; 4-HNE, 4-hydroxy-2-nonenal; JNK, c-Jun N-terminal kinase; Kcnip2, Kv channel interacting protein 2; L1cam, L1 cell adhesion molecule; Limk1, LIM domain kinase 1; MGST1, microsomal glutathione transferase 1; Mtch1, mitochondrial carrier homolog 1 *(C. elegans)*; Myh10, cellular myosin heavy chain, type B; NcoA6, nuclear receptor coactivator 6; Ngfr, nerve growth factor receptor; Ninj, ninjurin; Nrg1, neuregulin 1; Nrtn, neurturin; Pafah1b1, platelet-activating factor acetylhydrolase; Pax2, paired box 2; p38 MAPK, p38 mitogen activated protein kinase; Rac1, ras-related C3 botulinum toxin substrate 1; Rasgrf1, Ras protein-specific guanine nucleotide-releasing factor 1; Rtn4, reticulon 4; S100a6, calcyclin; Stmn1, stathmin 1; Tiam1, T-cell lymphoma invasion and metastasis 1; TNB, 5-thio-2-nitrobenzoic acid; Ywhah, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide

peroxidase activities and due to these unique properties plays a major role in biotransformation of xenobiotics, participates in the promotion of anticancer drug resistance, protects membranes from lipid peroxidation and their toxic products, mainly 4-HNE (Lee & DeJong, 1999; Yang *et al*., 2003; Johansson *et al*., 2007; Siritantikorn *et al*., 2007). MGST1 action is particularly important in the central nervous system since the age-related increase in reactive oxygen species is linked with the development of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, multiple sclerosis and amyotrophic lateral sclerosis (Uttara *et al*., 2009; von Bernhardi *et al*., 2010).

The aim of our work was to elucidate the role of MGST1 on the viability, morphology and antioxidant system of PC12 cells derived from a transplantable rat pheochromocytoma, a line that is also frequently used as a model for the study of neuronal functions.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. The PC12 rat pheochromocytoma cell line, heat-inactivated fetal bovine serum and heat-inactivated horse serum were obtained from ATCC (USA). RPMI 1640 medium (with 25 mM Hepes buffer, without L-glutamine) was from PAA (USA). Streptomycin and penicillin were from Polfa Tarchomin S.A. (Poland). Collagen type I from rat tail, L-glutamine, sodium pyruvate, D-(+) glucose solution 45%, BSA, 0.1% Tween 20, Sigma Fast BCIP/NBT, Glutathione Assay Kit, CDNB, DCNB, laminin, cocktail of proteases inhibitors, 4,6-diamidine-2-phenylindole (DAPI) and anti-goat IgG-FITC antibody were purchased from Sigma Aldrich (Germany). Total RNA Isolation Kit was from Epicentre Biotech. (USA). pCR®T7/NT-TOPO vector and pcDNA3.1(+) were from Invitrogen (USA). MGST1 and GAPDH primers and oligo(dT)$_{12\text{-}18}$ were from Institute of Biochemistry and Biophysics PAS (Warszawa, Poland). M-MLV reverse transcriptase was from Invitrogen Life Technologies (USA). dNTP was from Solis BioDyne (Estonia). SYBR Green Master Mix was purchased from Fermentas International Inc. (Canada). Nitrocellulose membrane 0.45 μm and Protein Assay Kit were from Bio-Rad Laboratories (USA). Primary antibodies against MGST1, β-actin, JNK, p-JNK, p38, pp-38, ERK1/2 and p-ERK1/2 were from Santa Cruz Biotechnology Inc. (USA). Annexin-V-FLUOS Staining Kit was from Roche Diagnostic GmbH (Mannheim, Germany). ArrayGrade™ Total RNA Isolation Kit, Neurogenesis and Neural Stem Cell Oligo GEArray (ORN-404), TrueLabeling-AMP 2.0 Kit and Chemiluminescent Detection Kit were from SABiosciences Corp. (Frederick, MD, USA). dUTP biotin was from Perkin Elmer (USA). G418 was from AppliChem GmbH (Germany). Trypan Blue was from Biowest LLC (USA). Vectashield® Mounting Medium was purchased from Vector Labs (USA). Eosin solution was purchased from PPH POCh S.A. (Poland). Hematoxylin solution was from AQUA-MEDICA (Poland).

Cell culture. PC12 cells were cultured on collagen (type I from rat tail) coated flasks in 85% RPMI 1640 supplemented with 25 mM Hepes buffer, 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 g/l D-(+)-glucose and antibiotics: 25 μg/ml streptomycin and 25 U/ml penicillin. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 atmosphere. The medium was exchanged every 48 h. Cells were rinsed with 1×PBS pH 7.0 and detached with 0.25% trypsin/EDTA. After centrifugation at 1000×*g* for 5 min cells were subcultured in 25 cm^2 flasks using subcultivation ratio of 1:2 to 1:4 and were photographed every 24 h with an inverted microscope (Olympus CK-40). Cells were passaged twice a week.

Plasmid construction and stable transfection. Vector carrying an antisense RNA sequence directed to MGST1 was constructed using standard recombinant DNA technologies. Briefly, cellular RNA was extracted from PC12 cells using Total RNA Isolation Kit according to the procedure provided by the manufacturer. One microgram of total RNA was then subjected to reverse transcription using oligo(dT)$_{12\text{-}18}$ primers. Amplified cDNA was used as a template to generate a 390-bp PCR product for MGST1 using the following primers: 5′-CGGATCCGCTCCTGAGCAGCCTGTAAGCAT-3′ and 5′-CGAATTCATTGAAAGCATGGCTGACCT-3′. PCR conditions were as follows: 95°C for 2 min, then 30 cycles of: 95°C for 30 s; 52°C for 60 s; 72°C for 90 s and the final extension at 72°C for 5 min. The PCR product was directly ligated to pCR®T7/NT-TOPO vector. The *Bam*HI/*Eco*RI restriction fragment containing 390 bp of MGST1 cDNA was subcloned in the antisense orientation in vector pcDNA3.1(+). The integrity and orientation of the insert was confirmed by restriction enzyme analysis and sequencing. Plasmid DNA was introduced into PC12 cells by electroporation and selection was initiated after 48 h. Stably transfected PC12_M cells were selected for antibiotic resistance with neomycin analog G418 at 800 μg/ml. PC12 cells transfected with empty plasmid, expressing neomycin resistance alone, was used as a control (PC12). Both lines were maintained in complete RPMI 1640 medium containing 200 μg/ml G418.

Quantitative real-time PCR. Total cellular RNA and cDNA were prepared as described previously (Zylinska *et al*., 2007). Real-time PCR was carried out on an Abi Prism™ 7000 sequence detection system (Applied Biosystems) using a SYBR Green master mix and cDNA obtained from each cell line. The following primers were used: MGST1 5′-ATTGAAAGCATGGCTGACCT-3′, 5′-GCTCCTGAGCAGCCTGTAAGCCAT-3′ and GAPDH 5′-GGTTACCAGGGCTGCCTTCT-3′, 5′-CTTCCCATTCTCAGCCTTGACT-3′. Amplification was performed by 10 min at 95°C followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. For each set, a melting curve was performed. Results are given as a relative expression rate of *mgst1* normalized to *gapdh* housekeeping gene, determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001).

Trypan Blue staining. Cell viability was determined using the Trypan Blue staining. Cells were harvested, suspended in PBS and mixed with equal volume of 0.5% Trypan Blue solution. After 5 min at room temperature, viable and dead (stained) cells were counted using a hemocytometer under an inverted microscope using a 10× magnification.

Flow cytometry analysis. Flow cytometry analysis was performed using a Becton Dickinson FACSCalibur and Annexin-V-FLUOS Staining Kit. Cells were harvested, washed with PBS and centrifuged at 1000×*g* for 5 min. Next, cells (10^4) were resuspended in 100 μl of Annexin-V-FLUOS labelling solution (100 μl Hepes buffer, 2 μl Annexin-V-Fluorescein and 2 μl propidium iodide) and incubated for 10–15 min at 15°C–25°C in the dark. The data were analyzed with Becton Dickinson CELLQuest software.

Immunocytochemistry. PC12 cells were grown on LabTek II CC2 chamber slides (Nunc, USA) coated with collagen (type I from rat tail). On the next day the medium was removed, cells were washed with PBS, fixed with 3.8% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 30 min and blocked with 6% BSA in PBS for 3 h at room temp. Then, the coverslips were incubated overnight at 4°C with primary anti-MGST1 antibodies (1:50) in 6% BSA in PBS. After several washes with PBS, cells were probed with FITC-conjugated anti-goat IgG secondary antibodies (1:400) for 2 h at room temp. Before mounting with Vectashield® Mounting Medium, cell nuclei were stained with DAPI. Microscopy was carried out with a confocal laser-scanning microscope TCS SP5 (Leica).

Migration assay. The migration assay was performed using Transwell Costar® chambers containing polycarbonate membranes (diameter — 6.5 mm) with 8 μm pores, which were placed in 24-well plates. The wells were coated with 10 μg/ml laminin in PBS, pH 7.4 for 2 h at 37°C and filled with serum-free RPMI 1640 containing 0.5% BSA. PC12 and PC12_M cells (1×10^6) in 100 μl serum-free RPMI 1640 containing 0.1% BSA were added to the chambers and allowed to migrate through the membranes under optimal conditions (37°C, 5% CO_2) for 4 h. After this time, cells found on the bottom side of the membranes were washed with PBS and fixed in 95% methanol. Next, the membranes were rinsed with PBS and stained with hematoxylin for 6 min and 0.25% eosin for 3 min at room temp. After staining they were washed with PBS and stained cells were counted under an inverted Nikon TMS-F microscope (magn. 20×). Non-specific migration was determined using 0.5% BSA as a control substrate.

Determination of glutathione content. Total glutathione (GSX) and glutathione disulfide (GSSG) was determined with Glutathione Assay Kit according to the manufacturer's protocol. Cells (3×10^5) were detached by trypsinization, centrifuged at $1000 \times g$ for 5 min and lysed with 10 mM HCl. Next, the pellets were frozen at –80°C and thawed at 37°C. The samples were deproteinized with 5% 5-sulfosalicylic acid and centrifuged at $8000 \times g$ for 10 min. Concentration of glutathione was determined in the supernatant. To block free thiols (–SH), 80 mM 4-vinylpyridine solution was added and incubated for 30 min at room temp. The amount of glutathione in the biological samples was determined from a standard curve of reduced glutathione and was presented as nmoles per mg of protein.

Preparation of postmitochondrial fraction. Cell cultures were rinsed twice with cold PBS and lysed for 30 min with ice-cold buffer containing: 10 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA and protease inhibitor cocktail: 1 mM PMSF, 1 μM pepstatin, and 10 μM leupeptin. The postmitochondrial fraction was obtained after centrifugation at $10\,000 \times g$ for 15 min at 4°C. Protein concentration was measured using Protein Assay kit.

Immunoblots. For Western blotting, 20–60 μg of proteins were separated on 15% SDS/PAGE followed by transfer to nitrocellulose. Membranes were blocked in TBS containing 3% BSA and 0.1% Tween 20 for 1–2 h and next incubated with primary antibodies: anti-MGST1 (1:1000), anti-MAP kinases: JNK, p-JNK, p38, p-p38, p-ERK1/2 (1:500) and ERK1/2 (1:750) for 12 h at 4°C. For staining, appropriate secondary antibodies (1:1000) coupled with alkaline phosphatase were used. Blots were developed with Sigma Fast BCIP/NBT according to the manufacturer's instructions. The intensity of bands was quantified by densitometric analysis using GelDoc ™EQ system with Quantity One software (Bio-Rad Laboratories, USA). Immunoblots were normalized to actin content with anti-actin antibody (1:3000).

Glutathione transferases activity. The determination of GSTs activity in postmitochondrial fraction was done after a 5 min incubation at 37°C with 1 mM CDNB and 1 mM GSH or 1 mM DCNB and 5 mM GSH as substrates, according to the procedure of Habig and Jakoby (1981). The activity was presented as nmoles per mg protein per minute.

Microarray analysis. Total RNA was isolated from 1×10^6 cell of both lines using ArrayGrade™ Total RNA Isolation Kit according to the procedure provided by the manufacturer. The concentration and purity of extracted RNA was determined spectrophotometrically at 260/280 nm. Synthesis, labeling and hybridization of cDNA samples to the GEArray Rat Neurogenesis and Neural Stem Cell Microarray (ORN-404-04) were performed according to the manufacturer's instruction. Hybridization and chemiluminescent detection were performed using Chemiluminescent Detection Kit according to the manufacturer's protocol. The imaging screens were scanned and analyzed with GEArray Expression Analysis Suite 2.0 (SuperArray Bioscience Corp.)

Statistics. Statistical analyses were done using the STATISTICA 8.0 Inc. (USA) computer program and $P < 0.05$ was considered to indicate statistically significant differences. Data presented are the mean of duplicate or triplicate determinations from at least four independent cell cultures.

RESULTS

To characterize the role of MGST1 in the functioning and development of PC12 cells we compared the control PC12 cell line (transfected with an empty plasmid) and PC12_M cells (with a reduced MGST1 level). Real-time PCR (Table 1) and Western blot analyses (Fig. 1) indicated that the expression of mRNA MGST1 and protein level decreased to 60% after transfection of PC12 cells with a plasmid containing the antisense target sequence. The first morphological changes in PC12_M cells were detected after 48 h (Fig. 2). These cells exhibited a stronger tendency to grow in clusters, weaker adhesion, a multipolar body of cells and displayed the formation of short neurite-like extension in comparison with the control PC12 line.

Viability of the cells estimated by Trypan Blue staining showed a higher percentage of dead cells in PC12_M (22%) than in the control line (6%). Decreased survival of PC12 cells with reduced MGST1

Table 1. Real-time PCR analysis of mRNA MGST1 level
The results are from three independent experiments. ΔCt, (average $Ct_{MGST1, PC12\_M}$ — average $Ct_{GAPDH, PC12\_M}$) for PC12_M or (average $Ct_{MGST1, PC12}$ — average $Ct_{GAPDH, PC12}$) for PC12; ΔΔCt (average $\Delta Ct_{MGST1, PC12\_M}$ — average $\Delta Ct_{MGST1, PC12}$); $2^{-\Delta\Delta Ct}$ — relative fold change of MGST1 mRNA level in PC12_M in relation to PC12.

	PC12	PC12_M
ΔCt	2.27 ± 0.17	3.10 ± 0.51
ΔΔCt	0.00	0.83 ±0.43
$2^{-\Delta\Delta Ct}$	1.00	**0.59 ± 0.16** *P = 0.000205*

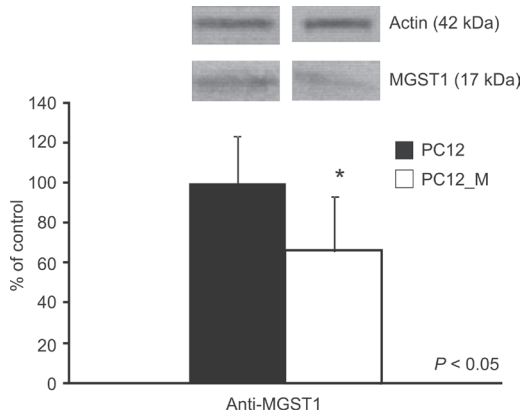

Figure 1. Western blot and densitometric analysis of MGST1 in postmitochondrial fractions of PC12 cell lines
Proteins were analyzed by SDS-polyacrylamide gel electrophoresis on 15% gels under reducing conditions followed by immunoblotting with anti-MGST1 or anti-actin. Quantification of MGST1 band was performed after densitometric scanning of immunoblots and normalized to actin level. The normalized amount of MGST1 in control PC12 cells was taken as 100%. Data shown are the mean from four independent experiments. *$P < 0.05$.

was confirmed by flow cytometry analysis that revealed a higher percentage of necrotic cells (Fig. 3; Table 2).

Immunocytochemical staining revealed differences in the concentration of MGST1 between the two cell lines (Fig. 4). The highest intensity of the fluorescent signal in the control line than in PC12_M cells confirmed the reduction of total MGST1 content in PC12_M. However, to determine specific distribution of MGST1 in the cells, further studies using appropriate intracellular markers are needed.

The possible role of MGST1 in tumor progression was analyzed by determining the ability of the cells to migrate. The cells were tested using two substrates — albumin as a control and laminin as a chemoattractant.

Table 2. Quantification of viable, apoptotic and necrotic cells in PC12 lines
Cell status was analyzed by FACS (distribution between different subpopulations of the cells in both cell lines is presented in percentage).

Cells	Viable	Early apoptosis	Late apoptosis	Necrosis
PC12	95.72	0.53	1.67	2.08
PC12_M	65.72	0.00	0.14	34.14

Table 3. Effect of MGST1 suppression on PC12 cells migration
Assays were performed using membrane filters coated with albumin (control substrate) or laminin (chemoattractant). Migration is expressed as the total number of cells that migrated across membrane during a 4 h incubation period.

Cells	Cells migration on albumin	Cells migration on laminin
PC12	51 ± 26	178 ± 41
PC12_M	57 ± 40	17 ± 2

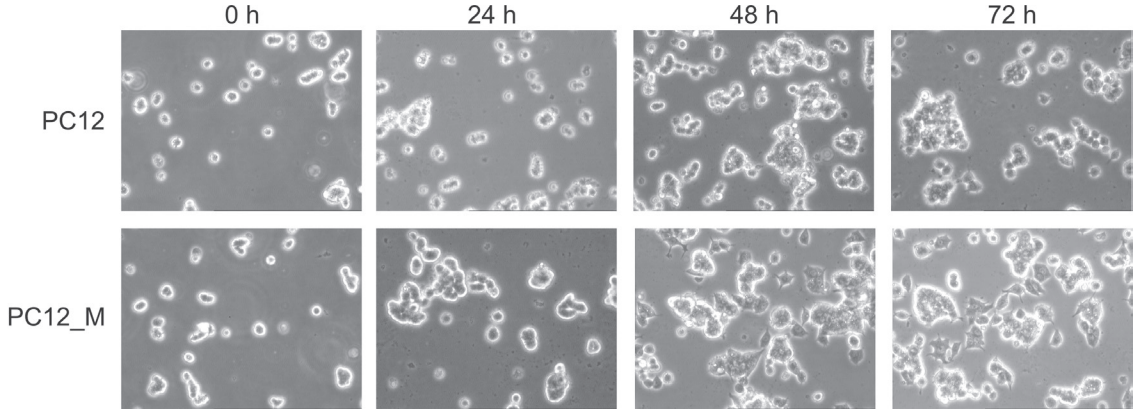

Figure 2. Morphological characteristic of PC12 cell lines
Cells were photographed after 24, 48 and 72 h of cultivation using an inverted Olympus CK-40 microscope at 20× magnification. PC12, control line transfected with empty plasmid; PC12_M, cell line transfected with plasmid containing anti-MGST1 sequence.

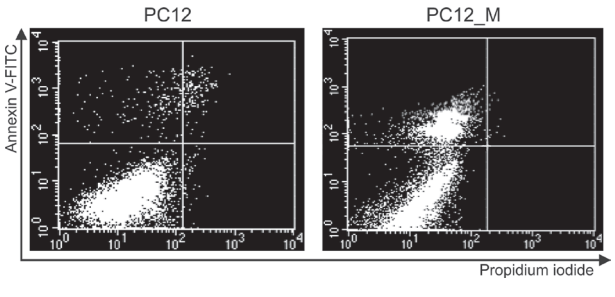

Figure 3. Flow cytometry analysis
FACS analysis was performed for 10^4 cells in each sample using double staining with Annexin V and propidium iodide, after 48 h of culturing. The lower left quadrant shows viable cells, lower right — early apoptotic cells, upper right quadrant — late apoptotic cells, upper left — necrotic cells.

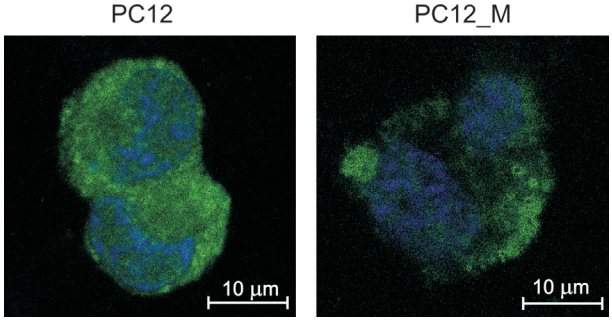

Figure 4. Immunocytochemistry of MGST1 in PC12 cell lines
Examination was performed with a confocal laser-scanning microscope TCS SP5 (Leica). Images represent overlapping of many optical sections. The blue signal is from cell nuclei stained with DAPI and the green signal from MGST1 stained with FITC-conjugated secondary antibodies.

Table 4. Glutathione concentration in PC12 cell lines
Concentration of reduced (GSH) and oxidized (GSSG) glutathione in PC12 cell lines was determined as described in Materials and Methods. Results are averages of four separate experiments performed in duplicate, using different cell cultures. *$P<0.05$ *vs.* control PC12 line.

Cells	GSX (nmoles/mg protein)	GSSG (nmoles/mg protein)	GSSG/GSH (%)
PC12	6.7 ± 1.5	0.3 ± 0.1	4.7
PC12_M	**8.8 ± 1.7***	0.2 ± 0.1	2.3

GSX, total glutathione; GSSG, disulfide glutathione; GSH, reduced glutathione

Both lines exhibited a similar potential for migration on the control substrate, whereas with laminin the control PC12 cells migrated three times more efficiently than the cells with reduced MGST1 (Table 3).

To examine the MGST1 influence on the antioxidant system we determined glutathione concentration. In PC12_M cells a statistically significantly higher total glutathione content was detected; however, the GSSG/GSH ratio was almost two-fold lower than in the control (Table 4).

To analyze the consequences of MGST1 suppression on the activity of cytosolic GSTs, which are an integral part of a dynamic and interactive cellular defense mechanism, in the next step the activity of glutathione transferases was assayed in the postmitochondrial fraction using CDNB, a non-specific substrate for all GSTs, and DCNB, which is specific only for cytosolic glutathione transferases. In the presence of CDNB the total activity of GSTs in PC12_M cells was lower by 15–20% from that in the control, whereas with DCNB the activity was similar in both examined lines (Fig. 5).

Based on the observed morphological and biochemical changes in the PC12_M line, we decided to characterize the activity of MAP kinases: p38, JNK and ERK1/2. Mitogen-activated protein kinases, an enzyme family involved in various cellular responses to external or endogenous signals, play a predominant role in stress response and in many normal physiological processes. Among others, the MAPK signalling pathway regulates cellular proliferation and differentiation, cell migration, apoptosis, and malignant transformation. In the cells with suppressed MGST1 the total protein content of p38, ERK1 and ERK2 was lower than in the control cells, but their phosphorylation level was higher. In contrast, for JNK the increase in a total protein content was accompanied by a lower intensity of phosphorylation (Fig. 6).

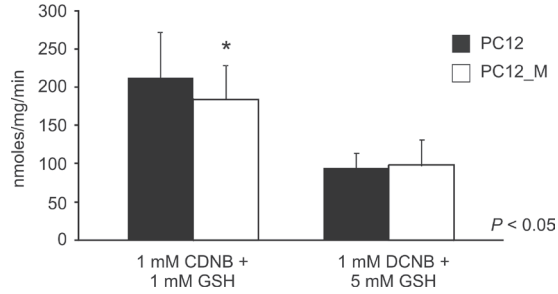

Figure 5. GSTs activity in postmitochondrial fraction
Samples were incubated with substrates at 37°C for 5 min and the activity was determined spectrophotometrically. The results are averages of four independent experiments performed in duplicate, using different cell cultures. *$P<0.05$.

To elucidate if the suppression of MGST1 could have any influence on the profile of gene expression, we performed a large-scale analysis using a commercially available microarray with selected 263 genes involved in neurogenesis, synaptic functions, cell cycle control, apoptosis, migration and motility, proliferation, differentiation and adhesion. Microarray experiments were repeated four times with representative images shown in Fig. 7. In PC12_M cells the expression of eight genes was higher and of eleven genes lower than in the control (Fig. 8). In both examined lines the microarray analysis also revealed several genes with similar high expression levels (*ache, bmp2, l1cam, ninj, nrg1, pafah1b1, rac1 and tiam1*). This suggests that the altered PC12_M metabolism did not affect their cellular function.

DISCUSSION

MGST1 has glutathione transferase and peroxidase activities and due to these specific properties plays a major role in detoxication of drugs and xenobiotics. It is also involved in anticancer drug resistance, may protect membranes against lipid peroxidation, mainly its toxic products such as 4-HNE, and participates in signaling pathways mediated by ROS — its substrates (Johansson *et al.*, 2007; Siritantikorn *et al.*, 2007, Lee *et al.*, 2008). This unique characteristic of MGST1 demonstrates its essential role in cell metabolism.

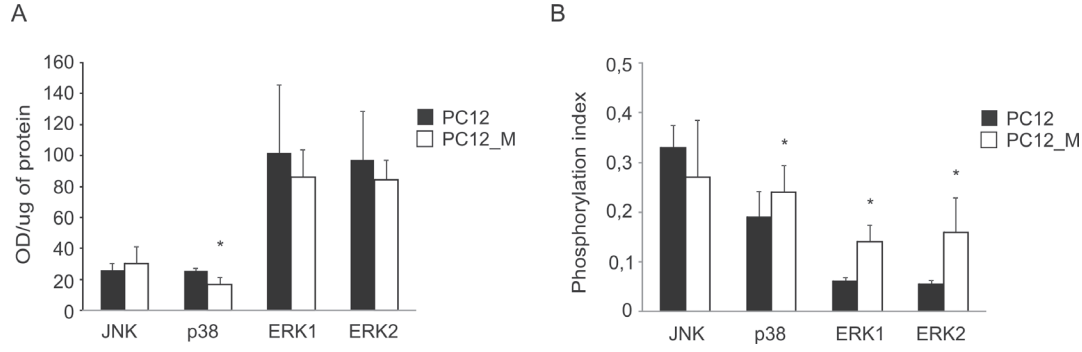

Figure 6. Analysis of MAP kinases in PC12 cell lines
The intensity of protein kinase bands was quantified by densitometric analysis of immunoblots. The results are averages of four independent experiments performed in duplicate, using different cell cultures. *$P<0.05$. **(A)** The amount of examined kinases was normalized to β-actin and is expressed in arbitrary units. **(B)** Phosphorylation index for each kinase is expressed as the ratio of normalized amount of phosphorylated kinase to the total amount of kinase protein.

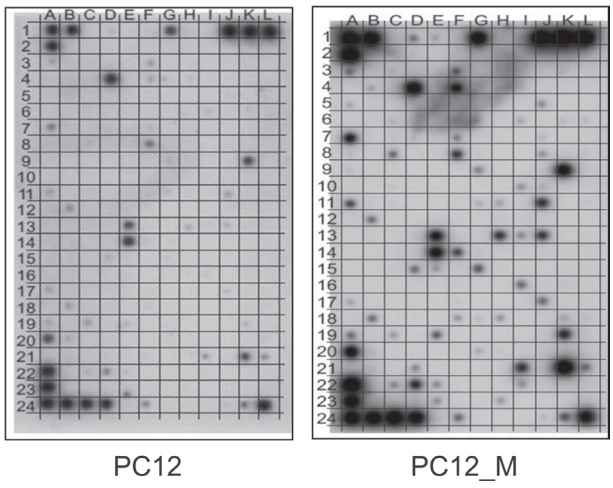

Figure 7. Microarray analysis of PC12 cell lines
Gene expression was analyzed using Rat Neurogenesis and Neural Stem Cell Microarray (ORN-404-04). Representative arrays are shown.

Enzymatic and non-enzymatic defense (such as glutathione) provide the first line of antioxidant protection against free radicals generated in the mitochondrial electron transport chain. Overproduction of ROS or diminished GSTs activity can initiate an autocatalytic increase in the membrane lipid peroxidation, resulting in the formation of a variety of toxic electrophilic species. These disturbances may be reflected at several levels, e.g., the cellular metabolism and the pattern of gene expression. To characterize the cellular effect of MGST1 suppression first we determined the total glutathione amount. A surprising observation was that the total glutathione content was higher in the PC12_M line than in the control, whereas the GSSG/GSH ratio — a good indicator of the cellular redox state was lower. This may suggests a reduced consumption of glutathione or its higher *de novo* biosynthesis. Glutathione participates in many cellular processes, such as antioxidant protection and nutrient metabolism, and is a regulator of cell division, apoptosis, signal transduction and protein glutathionylation (Wu *et al.*, 2004). Our results indicate that the antioxidant reserve of PC12_M cells is increased relative to the normal level and it could be a compensatory mechanism, which enables the survival of about 70% of the cells.

Mitochondria represent the major ROS source for the cell and posses unique antioxidant defense systems, including a specific pool of glutathione transferases (Jezek & Hlavata, 2005). It has also been shown that mitochondrial respiratory and redox functions are specifically affected under stress conditions (Raza & John, 2006). In the present study we analyzed the GSTs activity in the postmitochondrial fraction to discriminate the effect of MGST1 suppression on the GSTs activity between mitochondria and other cellular compartments. In PC12_M cells a decreased total GSTs activity towards CDNB was noticed, whereas with DCNB as a substrate, the activity was similar to that in the control line. This suggests that the decline in MGST1 cannot be compensated by an increase of the activity of other cytosolic GSTs and it underlines the exceptional function of this enzyme. Further detailed study is needed to elucidate the mitochondrial participation in the observed effects.

Although the reduction of MGST1 increased the overall level of necrotic cells, it simultaneously triggered differentiation of the remaining PC12_M cells. Rat pheochromocytoma PC12 cells upon exposure to neurotrophin or dibutyryl-cAMP can be induced to differentiate into sympathetic neurons (Lambeng *et al.*, 2001). In PC12_M line we observed an altered cell morphology — from that typical for cancer cells to pseudoneuronal one. However, the observed neurite outgrowth without any external differentiation factors was distinctly different from that after neurotrophin stimulation reported by several studies. Preliminary analyses using a commercially available microarray showed that MGST1 suppression changed the profile

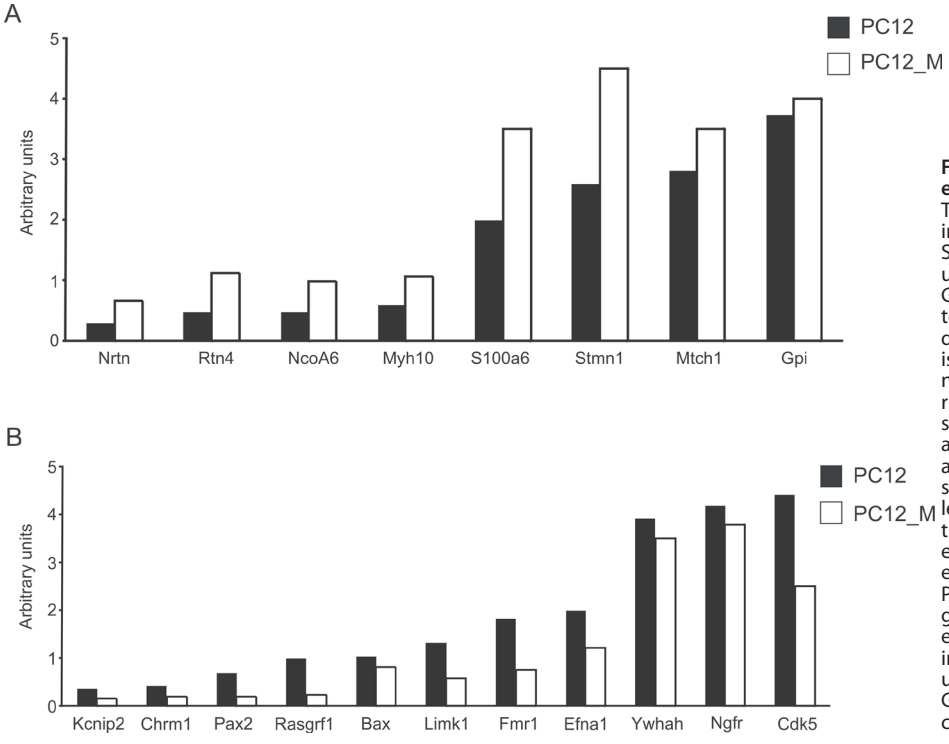

Figure 8. Comparison of gene expression in PC12 cell lines
The analyses were performed using Rat Neurogenesis and Neural Stem Cell Microarray as described under Materials and Methods. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A, lactate dehydrogenase A, ribosomal protein L32, 5S rRNA gene, aldolase A and ribosomal protein S27a. After intra- and inter-array standardization and normalization, genes demonstrating undetectable expression levels in at least half of the control or experimental groups were eliminated. Significant differences between control PC12 and PC12_M were observed for 19 genes ($P < 0.05$). Bars indicate the expression level of a given gene in examined cell lines. **(A)** Genes up-regulated in PC12_M cells. **(B)** Genes down regulated in PC12_M cells.

of genes expression. These alterations could be relevant to the survival, change in a phenotype and modification of signaling pathways in PC12_M cells, particularly crucial for neurogenesis and synaptic processes. At present it is difficult to explain conclusively this phenomenon, but we detected elevated expression of *nrtn* gene, encoding a neurotrophin with a strong effect on neurite outgrowth (Oiwa *et al*., 2002).

Another potentially interesting observation was the increased expression of *stmn1*, which is present in a high amount in the central nervous system and plays a significant role during NGF-stimulated differentiation of PC12 cells, and is also involved in cell proliferation (DiPaolo *et al*., 1996; Mistry & Atweh, 2002). The outgrowth of small neurites in PC12_M cells could be due to increased expression of *rtn4* gene — a potent inhibitor of axonal growth. The Rtn4 protein, by interacting with adhesion molecules like integrins, decreases the cells ability to adhere to the substrate (Yang & Strittmatter, 2007; Hu & Strittmatter, 2008). Also another protein, Efna1, which regulates dendrite morphology and formation of new neuronal connections (Wykosky & Debinski, 2008) was expressed in PC12_M cells at a diminished level.

Necrosis is often defined as uncontrolled process of cell death, but recent studies have shown that cells can change the type of death from apoptosis or autophagy to necrosis. It could be linked with inhibition or lowered expression of regulatory proteins required for apoptosis such as Bax (Golstein & Kroemer, 2006). Apoptosis of neuronal cells depends on the cellular balance between pro-apoptotic (Bax, Box, Bad) and anti-apoptotic proteins (Bcl-2, Bcl-x, Bcl-w) (Tehranian *et al*., 2008). The observed higher amount of necrotic cells in the PC12_M line might result from disturbances in the control of Bax-regulated apoptosis. Moreover, this process could also be enhanced by the apparent decrease in phosphorylation level of c-Jun N-terminal kinase (JNK). The enzyme is responsible for regulation of transcription of several genes related to apoptosis, including *bax* and 14-3-3 protein family (Wagner & Nebreda, 2009). It is notable that one of these proteins is Ywhah, whose diminished expression at the transcript level was detected in PC12_M. We also found an altered expression of two other genes coding for proteins that regulate apoptotic events — Ngfr, a pro-apoptotic receptor, and Rtn4, which binds to proteins from the Bcl2 family and inhibits their anti-apoptotic activity (Yang & Strittmatter, 2007; Bertrand *et al*., 2008). An interesting observation was the increased expression of S100a6 (calcyclin) in the PC12_M line. The gene for this calcium binding protein has an ARE sequence (antioxidant responsive element) in the promoter region and the protein plays an important role in protecting cells from oxidative stress (Filipek *et al*., 2007). Some data have demonstrated differential effects of S100A6 on apoptosis, which depend on the specific cell type and preferential activation of either pro- or anti-apoptotic signaling pathways. Thus, the final result of these regulations appears to be governed by the integration of survival and death signals.

Cell migration is crucial in multiple physiological processes including nervous system development — axon extension, synapse formation, nerve regeneration after injury as well as wound healing, angiogenesis, inflammation, tumor invasion and metastasis (Tomaselli *et al*., 1987; Rankin *et al*., 2006). A strong positive correlation between level of glutathione-*S*-transferase Pi expression and increased metastasis was shown in primary gastric cardiac adenocarcinoma (Shi *et al*., 2008). In our model, the suppression of MGST1 significantly reduced the ability of cells to migrate. One of the causes could be a diminished expression of Limk1, a kinase that plays a critical role in phosphorylation of the cofilin family proteins (Scott & Olson, 2007). Limk1 overexpression in human prostate and breast cancer cell lines correlated with higher levels of both phosphorylated cofilin and metastasis (Davila *et al*., 2003; Bagheri-Yarmand *et al*., 2006). *L1cam* also regulates intercellular interactions and participates in adhesion and migration processes (Raveh *et al*., 2009). An additional regulatory factor could be modification of the MAP kinases pathway, especially JNK and ERK1/2, which has been shown to induce the expression of some proteins involved in metastasis (Kim & Choi, 2010). The altered migration, morphology and enhanced necrosis in PC12_M could be a result of a concerted action of all genes that act as regulators of cytoskeletal elements, synaptogenesis and cell survival.

Of the genes engaged in regulation of migration, we observed the disparity between *nrg1, pafah1b1, myh10* and *cdk5* expression in PC12_M line. However, whereas the level of *myh10* was increased, *cdk5* was down-regulated and *nrg1* and *pafah1b1* were expressed at a similar level as in control cells. Cdk5, a very effective serine/threonine kinase, has been shown to phosphorylate a spectrum of proteins, mostly those associated with cell morphology and motility (Tripathi *et al*., 2010). Moreover, deregulation of Cdk5 has been implicated in several neurodegenerative diseases (for a review see Dhariwala & Rajadhyaksha, 2008). The changed expression of other genes necessary for neurogenesis and synaptic transmission — *mtch1, gpi, rasgrf1, kcnip2, chrm1, fmr1, ywhah, tiam1, ache* — as well as of a group of genes that regulate cell proliferation and cell cycle — *pafah1b1, rasgrf1, ywhah* and *bax* — clearly indicates that suppression of MGST1 triggered complex gene expression changes. Elucidation of the exact molecular mechanisms of these changes needs further detailed study.

Taken together, our results suggest that activity of MGST1 could be an important regulator of PC12 cells development. Understanding the mechanisms by which MGST1 can influence the expression of genes with different biological functions is of primary importance for the design of new therapeutic approaches to cancerogenesis and neurodegenerative diseases.

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