Impact of Elastin-Derived Peptide VGVAPG on Matrix Metalloprotease-2 and -9 and the Tissue Inhibitor of Metalloproteinase-1, -2, -3 and -4 mRNA Expression in Mouse Cortical Glial Cells In Vitro

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Abstract
Degradation products of elastin, i.e. elastin-derived peptides (EDPs), are involved in various physiological and pathological processes. EDPs are detectable in cerebrospinal fluid in healthy people and in patients after ischemic stroke. However, to date, no studies concerning the role of EDP in the nervous system were conducted. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play important roles during the repair phases of cerebral ischemia, particularly during angiogenesis and reestablishment of cerebral blood flow. Therefore, the aim of this study was to investigate the impact of the specific elastin-derived peptide VGVAPG on Mmp-2, -9 and Timp-1, -2, -3 and -4 mRNA expression in mouse cortical glial cells in vitro. Primary glial cells were maintained in DMEM/F12 without phenol red supplemented with 10% fetal bovine serum and the cells were exposed to 50 nM, 1 and 50 μM of the VGVAPG peptide. After 3 and 6 h of exposition to the peptide, expression of Mmp-2, -9 and Timp-1, -2, -3 and -4 mRNA was measured. Moreover, siRNA gene knockdown, cytotoxicity and apoptosis measurement were included in our experiments, which showed that VGVAPG in a wide range of concentrations exhibited neither proapoptotic nor cytotoxic properties in mouse glial cells in vitro. The peptides enhanced mRNA expression of Timp-2 and Timp-3 genes in an elastin-binding protein (EBP)-dependent manner. However, changes in mRNA expression of Mmp-2, Mmp-9 and Timp-4 were partially EBP-dependent. The decrease in mRNA expression of Timp-1 was EBP-independent. However, further studies underlying the VGVAPG peptide’s mechanism of action in the nervous system are necessary.

Keywords Elastin-derived peptides • VGVAPG • Glial cells • MMP-2 • MMP-9 • TIMPs

Highlights
• The VGVAPG peptide in a wide range of concentrations exhibited neither proapoptotic nor cytotoxic properties in mouse astrocytes in vitro
• The VGVAPG peptide enhanced mRNA expression of Timp-2 and Timp-3 genes in an elastin-binding protein (EBP)-dependent manner
• The VGVAPG peptide’s change in the mRNA expression pattern of Mmp-2, Mmp-9 and Timp-4 was partially EBP-dependent
• The VGVAPG peptide’s decrease in the mRNA expression of Timp-1 was EBP-independent

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Abbreviations
CNS Central nervous system
CSF Cerebrospinal fluid
DMSO Dimethyl sulfoxide
EDPs Elastin-derived peptides
FasR Fas receptor
FBS Fetal bovine serum
LDH Lactate dehydrogenase
PBS Phosphate-buffered saline
ROS Reactive oxygen species
MMP Matrix metalloproteinase
TIMP Tissue inhibitor of metalloproteinase
VGVAPG Val-Gly-Val-Ala-Pro-Gly
siRNA Small interfering RNA
EBP Elastin-binding protein
Introduction

Elastin is an essential protein in mammalian organisms and provides elasticity to many connective tissues such as the major arteries, lung, cartilage, elastic ligaments and skin. Products of proteolytic degradation of elastin, namely elastin-derived peptides (EDPs), are involved in various physiological and pathological processes (Gmiński et al. 1992, 1993). EDPs are detectable in cerebrospinal fluid (CSF) in healthy people (6.3 ng/mL) and in patients after ischemic stroke (129.5–205.0 ng/mL) (Nicoloff et al. 2008; Crocker et al. 2004), which suggests involvement of EDPs in pathological conditions and/or the regeneration process. In different physiological and pathophysiological conditions, such as inflammation or atherosclerosis, elastin is prone to proteolytic degradation which frees Val-Gly-Val-Ala-Pro-Gly (VGVAPG)-containing fragments (Lombard et al. 2006; O’Rourke 2007). The VGVAPG (498.58 molecular weight (MW)) hexapeptide is repeated multiple times in elastin molecules and binds to 67-kDa elastin-binding protein (EBP) with high affinity (Blood et al. 1988; Senior et al. 1984). EBP is a catalytically inactive form of beta-galactosidase produced by alternative splicing of the GLBI gene (Hinek et al. 1993; Skeie et al. 2012). To date, it has been demonstrated that VGVAPG induces diverse biological effects through EBP, depending on the research model. The VGVAPG peptide induced normal human cell proliferations such as fibroblast, monocyte and cancerous, e.g. human astrocytoma (Jung et al. 1998; Senior et al. 1984). Furthermore, the VGVAPG peptide exhibits strong chemotactic properties in the murine lung carcinoma cell line (M27) and facilitates the invasion of human melanoma cells (WM35 and HT168-M1) (Blood et al. 1988; Pocza et al. 2008). In addition to its chemotactic properties, it has been shown that EDPs or the VGVAPG peptide also upregulated the expression of different metalloproteinases (Floquet et al. 2004; Siemianowicz et al. 2010).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular matrix-degrading enzymes involved in diverse homeostatic and pathological processes (Agrawal et al. 2008; Crocker et al. 2004). MMP-2 and MMP-9 (gelatinase A and B, respectively) are expressed within the central nervous system (CNS) and perform important normal and pathological functions during development and adulthood (Crocker et al. 2004; Yong et al. 2001). A number of papers show the emerging roles of MMP-2 and MMP-9 and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs) in the regulation of astrocytic and neuronal cell death (Cunningham et al. 2005). In addition, MMPs and TIMPs are likely to play important roles during the repair phases of cerebral ischemia, particularly during angiogenesis and reestablishment of cerebral blood flow (Cunningham et al. 2005; Vanmeter et al. 2001; Wang et al. 2014). These processes will have important implications for therapies using MMP inhibitors in stroke (Cunningham et al. 2005). To date, it has been shown that the VGVAPG peptide in concentrations of 100 ng/mL ≈ 200.57 nM and 200 ng/mL ≈ 401.14 nM enhances angiogenesis by promoting endothelial cell migration and tubulogenesis through upregulation expression of mRNA of membrane-type matrix metalloprotease-1 (MT1-MMP) and MMP-2 (Robinet 2005). A similar result was obtained by Ntai et al. (2004), who showed that cell culture plates coated with 100.28 or 401.14 μM of VGVAPG caused an increase in the expression and activation of MMP-2 and MT1-MMP in two melanoma (M1Dor and M3Da) cell lines. Furthermore, it was shown that adding 200 μg/mL ≈ 401.14 μM of the VGVAPG peptide to the culture medium upregulated MMP-2, MT1-MMP and TIMP-2 mRNA expression and activity in the human fibrosarcoma (HT-1080) cell line and thus increased invasiveness of HT-1080 cells (Brassart et al. 1998; Donet et al. 2014).

Data concerning the VGVAPG peptide in CNS are very poor and limited to a few publications. So far, it has been demonstrated that 200 nM of the VGVAPG peptide can stimulate dendrite formations in mouse primary neuron culture (Chang et al. 2008). Furthermore, in human glioblastoma multiforme cell lines CB74, CB109 and CB191 and the rat astrocytoma cell line C6 exposed to 500 ng/mL ≈ 334.28 nM of the (VGVAPG)3 peptide, mRNA expression of MMP-2 dramatically increased with very low stimulation of MMP-9 (Coquerel et al. 2009). The authors linked this high expression of MMP-2 mRNA with an increasing number of migrating cells. Even though EDPs have been detected in ageing brains and different pathologies of the CNS, no studies on EDPs’ role on normal glial cells have been conducted so far.

The aim of this study was to investigate the impact of specific elastin-derived peptide Val-Gly-Val-Ala-Pro-Gly (VGVAPG) on matrix metalloprotease-2 and -9 (Mmp-2, -9) and the tissue inhibitor of metalloproteinase-1,-2,-3 and -4 (Timp-1, -2, -3 and -4) mRNA expression in mouse cortical glial cells in vitro.

Materials and Methods

Reagents

DMEM/F12 1:1 (16-405-CVR) without phenol red was purchased from Corning (Manassas, USA). Trypsin, streptomyacin, penicillin, glycerol, CHAPS, HEPES, dithiothreitol (DTT), NaCl, EDTA, calcein AM, Hoechst 33342 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The substrate for caspase-3 (235400) was purchased from Merck (Darmstadt, Germany). The cytotoxicity detection kit and FastStart Universal Probe Master (Rox) were purchased from ROCHE Applied Science (Mannheim, Germany). The Glib1 gene siRNA (sc-61342)
was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The VGVAPG peptide was synthesised by Lipopharm.pl (Gdańsk, Poland). Charcoal/dextran-treated fetal bovine serum (FBS) was purchased from EURx (Gdańsk, Poland). The cDNA reverse transcription kit – High Capacity cDNA – Reverse Transcription Kit and the TaqMan® probes corresponding to specific genes encoding Actb (Mm00607939_s1), Mmp-2 (Mm00439498_m1), Mmp-9 (Mm00442991_m1), Timp-1 (Mm01341361_m1), Timp-2 (Mm00441825_m1), Timp-3 (Mm00441826_m1) and Timp-4 (Mm01184417_m1) were obtained from Life Technologies Applied Biosystems (Foster City, CA, USA). Stock solutions of the VGVAPG peptide were prepared and added to DMEM/F12 medium. The final concentration of DMSO in the culture medium was always 0.1%.

**Glial Cell Culture**

The experiments were performed on mouse glial cells cell culture isolated from the fetuses (17/18 embryonal day) of pregnant female Swiss mice. Animals were anesthetised with CO₂ vapour and killed by cervical dislocation. All procedures were approved by a Bioethics Commission (no. 46/2014, First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow), as compliant with European Union law. After isolation and digestion process, cells were centrifuged and the pellet was suspended in DMEM/F12 1:1 without phenol red supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.10 mg/mL streptomycin and 250 ng/mL amphotericin B with modifications of the previously described method (Blomstrand and Giaume 2006; Vitvitsky et al. 2006; Wang et al. 1998), [see different glial culture media and the techniques review in Saura (2007)]. The cells were seeded at a density of 20 × 10⁶ cells/75 cm² in culture flasks. The cultures of the glial cells were maintained at 37 °C in atmosphere containing 5% CO₂. In the logarithmic phase, after reaching 90% confluence, the cells were collected and frozen in liquid nitrogen. This procedure kills neurons in culture and leaves the glial cells, which allows to collect a large number of cell banks and to store cells for further research. Before the experiment, the cells were thawed and seeded in culture flasks and cultured for an additional 24 h. After this period, glial cells were lysed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT) in 10 °C for 10 min. After initial incubation, the lysates were incubated with caspase-3 substrate Ac-DEVD-pNA at 37 °C. Cells treated with 1 μM staurosporine were used as a positive control (result not shown). After 30 min, absorbance of the lysates at 405 nm was measured using a microplate reader (FilterMax F5 Multi-Mode microplate reader). The amount of colorimetric product was continuously monitored for 120 min.

**Hoechst 33342 and Calcein AM Staining**

Hoechst 33342 and calcein AM staining were used to determine DNA fragmentation and metabolic activity and/or cell viability of cultured glial cells. Mouse primary glial cells were exposed to 1 μM of the VGVAPG peptide and the cells were cultured for an additional 24 h. After this period, glial cells were stained according to previously described method (Szychowski et al. 2015). The cells were washed with phosphate-buffered saline (PBS) and exposed to Hoechst 33342 and calcein AM diluted in DMEM/F12 1:1 and added to the glial cells culture at a final concentration of 10 and 4 μM, respectively. The cells were incubated for 15 min in an atmosphere of 5% and CO₂ at 37 °C, washed one time in PBS and visualised by using a fluorescence microscope (LSM 700, ZEISS). Cell fluorescence in microphotographs was quantified by densitometry using ImageJ 1.50b software (National Institutes of Health, Bethesda, USA).

**siRNA Gene Silencing Procedure**

Glb1 siRNA was used to inhibit Glb1 gene expression in mouse primary glial cells. siRNA was applied for 7 h at a final concentration of 50 nM in antibiotic-free medium containing the siRNA transfection reagent INTERFERin, according to previously described method (Szychowski et al. 2017). The effectiveness of Glb1 mRNA silencing with the use of 50 nM specific siRNA was verified by measuring the mRNA levels. Cells were treated with 1 μM of the VGVAPG peptide and after 3 and 6 h, expression of genes encoding Mmp-2, Mmp-9, Timp-1, Timp-2, Timp-3 and Timp-4 was determined.
Real-Time PCR Analysis of mRNAs Specific to Genes Encoding Mmp-2, Mmp-9, Timp-1, Timp-2, Timp-3 and Timp-4

For the real-time PCR assay, glial cells were seeded on 12-well plates and initially cultured for 24 h. After 3 and 6 h of exposure to 50 nM and 1 and 50 μM of VGVAPG, samples of total RNA were extracted from glial cells according to the manufacturer’s protocol based on a previously described method (Szychowski et al. 2017). After siRNA transfection, the procedure was performed after 3 and 6 h of exposure for a concentration of 1 μM of VGVAPG. The RNA quality and quantity were determined spectrophotometrically at 260 nm and 280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). Two-step real-time RT-PCR was conducted using the CFX Real Time System (BioRad, USA). The reverse transcription (RT) reaction was performed at a final volume of 20 μL with 180 ng of RNA (as a cDNA template) using the cDNA reverse transcription kit according to the manufacturer’s protocol. Products from the RT reaction were amplified using the FastStart Universal Probe Master (Rox) kit with TaqMan probes as primers for specific genes encoding Actb, Mmp-2, Mmp-9, Timp-1, Timp-2, Timp-3 and Timp-4 according to the manufacturer’s protocol. Amplification was carried out in a total volume of 20 μL containing 1.5 μL of the RT product, and β-actin was used as the reference gene.

Statistical Analysis

The data are presented as the means ± SD of three independent experiments. Each treatment in experiment was repeated six times (n = 6) and measured in triplicate. The average of the triplicate samples was used for the statistical analyses. Statistical analysis was performed on the original results. Considering the different data from the measurement of absorbance, the results were presented as a percentage of controls (LDH and caspase-3 measurements). The data were analysed via one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison procedure in STATISTICA 10 software.

Results

Impact of VGVAPG on Cell Viability Measurements

LDH Cell Viability Assay

After 24 and 48 h of exposure of primary mouse glial cells to the studied VGVAPG peptide in concentrations ranging from 100 pM to 100 μM, we observed a lack of stimulation of LDH release (Fig. 1a).

Caspase-3 Activity

After 24 and 48 h of exposure of primary mouse glial cells to the studied VGVAPG peptide in concentrations ranging from 100 pM to 100 μM, we observed a lack of stimulation of caspase-3 activity (Fig. 1b).

Cell Staining

Primary mouse glial cells were stained with Hoechst 33342 and calcein AM to determine the cell number and to assess their viability and/or metabolic activity. After 24 h of exposition of the cells to 1 μM of the VGVAPG peptide, no changes in the number of cells and their viability in comparison to the control cells were detected (Fig. 2).

Impact of VGVAPG on mRNA Expression Levels in Mouse Cortical Glial Cells

Expression of Mmp-2 and Mmp-9 mRNA

Primary mouse glial cells were exposed to 50 nM, 1 and 50 μM of VGVAPG for 3 and 6 h. After 3 h of exposure to all VGVAPG peptide concentrations, we observed no change in Mmp-2 or Mmp-9 mRNA expression as compared to the control (Fig. 3a). However, after 6 h, there was a decrease in mRNA expression of Mmp-2 and Mmp-9 in cells exposed to 50 nM and 1 μM of VGVAPG as compared to the control (Mmp-2 mRNA expression decreased by 17.7 and 16.3% respectively as compared to the control; Mmp-9 mRNA expression decreased by 33.90 and 44.90% respectively as compared to the control) (Fig. 3b).

Expression of Timp-1, Timp-2, Timp-3 and Timp-4 mRNA

After 3 h of exposure to 1 and 50 μM of VGVAPG, a decrease in Timp-1 mRNA expression by 70.20 and 69.20% was observed as compared to the control. In the same time period, 50 nM and 1 μM of VGVAPG decreased Timp-3 mRNA expression by 25.60 and 32.67%, respectively, as compared to the control. Moreover, in cells exposed to 1 and 50 μM of VGVAPG, expression of Timp-4 mRNA increased by 140.66 and 147.00%, respectively, as compared to the control (Fig. 4a).

After 6 h of exposure to 1 μM of VGVAPG, no changes in Timp-1 mRNA expression were observed. Moreover, for all of the studied concentrations of VGVAPG (50 nM and 1 and 50 μM), an increase in Timp-2 mRNA expression by 36.80, 41.50 and 20.30%, respectively, was observed as compared to the control. Furthermore, concentrations of 50 nM and 1 μM of the VGVAPG peptide increased expression of Timp-3 by 22.70 and 26.30%, respectively, as compared to the control. However, 1 μM of VGVAPG decreased Timp-4 mRNA expression by...
19.70% as compared to the control. Interestingly, 50 μM VGVAPG increased Timp-4 mRNA expression by 22.60% as compared to the control (Fig. 4b).

Impact of VGVAPG on mRNA Expression Levels After Silencing the Glb1 Gene by siRNA in Mouse Cortical Glial Cells

After the Glb1 gene silencing procedure, 3 h of exposure to 1 μM of VGVAPG decreased mRNA expression of Mmp-2 and Mmp-9 (decreased by 77.00 and 37.40%, respectively) as compared to the Glb1 siRNA control. However, the use of Glb1 siRNA alone caused a decrease in the expression of Mmp-9 mRNA as compared to the cells with scramble siRNA (decrease by 23.60%, as compared to the scramble control). The expression of Timp-1 and Timp-2 mRNA genes did not change as compared to the scramble-siRNA control (Fig. 6b).

Discussion

Our study investigated the elastin-derived peptide, VGVAPG, mechanism of action in mouse primary glial cells in vitro. It is known that peptides liberated from a degraded extracellular matrix may interact with different receptors on the cell surface which results in the activation of intracellular signalling pathways which can lead to cellular events as diverse as cell adhesion, migration, proliferation, protein synthesis or apoptosis (Maquart et al. 2004). Due to a lack of data concerning the VGVAPG peptide mechanism of action in CNS, first we investigated its cytotoxic or proapoptotic potential. Our data showed that the VGVAPG peptide was not cytotoxic and did not activate apoptotic cell death in a broad spectrum of concentrations (100 pM to 100 μM) and in all of the studied time intervals (24 and 48 h) in mouse primary glial cells in vitro. A lack of apoptosis is important because the number of EDPs highly increases after a stroke and is accompanied by an inflammation process that can finally lead to cell death (Jin et al. 2010; Nicoloff et al. 2008; Tzvetanov et al. 2008). Moreover, in cells stained with Hoechst 33342 and calcein AM dyes, we observed no changes in the cell morphology and cell viability after 24 h of exposition of primary glial cells in vitro to 1 μM of the VGVAPG peptide.

Our studies are the first to demonstrate that after 3 h of exposure to 50 nM, 1 and 50 μM of the VGVAPG peptide, mRNA expression of Mmp-2 and Mmp-9 did not change. After 6 h of exposure to 50 nM and 1 μM of the VGVAPG peptide, Mmp-2 and Mmp-9 mRNA expression decreased as compared to the control level. Interestingly, mRNA expression of both of the studied genes did not change in a concentration of 50 μM of VGVAPG. There are no data concerning MMP-2 and MMP-9 mRNA expression after stimulation of the VGVAPG peptide in normal cells derived from mouse or human CNS. The available data concerning cancer cells derived from the nervous system show that 100 ng/mL ≥
66.86 nM of the (VGVAPG)₃ peptide strongly upregulated MMP-2 mRNA expression in the rat astrocytoma cell line C6 and human gliomas, such as CB74, CB109 and CB191 (Coquerel et al. 2009). In the same paper, the authors showed a very weak MMP-9 mRNA expression in two of the four studied gliomas. To date, Mmp-2 and Mmp-9 mRNA and protein upregulation by the VGVAPG peptide are well documented in different cell culture models, but mainly in cancerous ones. The discrepancy between our results and mentioned data could be the result of normal and cancer cells differences. The possible mechanism of the decrease in MMP-2 and MMP-9 in glial cells could be connected with nitric oxide (ON) pathways. There has been shown a link between NO and MMP-2 and MMP-9 mRNA expression. Such interaction was previously detected in humane endothelial cells in which NO caused the decrease in expression of both MMP-2 and MMP-9 mRNA (Chen and Wang 2004; Phillips and Birnby 2004). Moreover, a similar association has been shown in rat primary astrocytes, where downregulation of Mmp-9 mRNA levels was caused by NO (Shin et al. 2007). It is known that the VGVAPG peptide has high affinity to EBP (Devy et al. 2010; Robinet et al. 2005; Senior et al. 1984). Therefore, we decided to use specific siRNA targeting in the Glb1 gene which is alternatively spliced in EBP. In glial cells with Glb1 gene
knockdown, 3 h of exposure to VGVAPG decreased Mmp-2 and Mmp-9 mRNA expression. The obtained data suggest that Mmp-2 and Mmp-9 mRNA expression can be partially dependent on EBP activation.

After secretion from producing cells, MMP activity can be regulated by TIMPs (Kurzepa et al. 2014). TIMP-1 inhibits most of the MMPs with the highest affinity to MMP-9, but TIMP-2 and TIMP-4 have the highest affinity to MMP-2 (Brew et al. 2000; Trojanek 2015). TIMP-3 is different than the other TIMPs, as it is insoluble, attached to the extracellular matrix (ECM) and inhibits disintegrin and metalloproteinase (ADAMs) with high affinity (Crocker et al. 2004). However, TIMPs also perform other physiological functions. TIMP-1 and TIMP-2 are responsible for such functions as induction of erythropoiesis, mitogenesis and apoptosis (Docherty et al. 1985; Guedez et al. 1998b; Stetler-Stevenson et al. 1992). Moreover, astrocytic Timp-1 promotes oligodendrocyte differentiation and enhances myelination in CNS (Moore et al. 2011). TIMP-2 inhibits endothelial cell growth induced by fibroblast growth factor (Ahonen et al. 2003; Lipka and Boratyński 2008). TIMP-3 also shows proapoptotic properties due to stabilisation of the Fas receptor (FasR), while TIMP-1 and TIMP-2 can also exhibit anti-apoptotic properties (Cawston and Mercer 1986; Guedez et al. 1998a). Moreover, an increase in mRNA expression of both Timp-2 and Timp-3 has been observed in places of increased neurogenesis, cell
maturation and migration (Jaworski and Fager 2000; Vaillant et al. 1999). To date, the role of TIMP-4 continues to be poorly understood, although it has been shown that TIMP-4 activated apoptosis of fibroblasts from cardiac muscle and inhibited migration of cells capable of forming capillaries, while there was no impact on the process of proliferation and angiogenesis (Koskivirta et al. 2006).

Our data are the first to show that after 3 h of exposure to 1 and 50 μM of the VGVAPG peptide, Timp-1 mRNA expression in glial cells was significantly decreased. However, after 6 h of exposure to 1 and 50 μM of the VGVAPG peptide, the Timp-1 mRNA expression pattern did not change as compared to the control. Silencing of the Glb1 gene did not change the Timp-1 mRNA expression pattern caused by 1 μM of the VGVAPG peptide. Such data suggest that in Timp-1, mRNA expression is regulated by an EBP-independent mechanism.

To date, it has been shown that after birth in the rat brain, Timp-1 mRNA expression significantly decreased (Fager and Jaworski 2000). Similarly, downregulation of Timp-1 mRNA and protein expression was observed in Sprague Dawley rats with ischemic-reperfusion (Ma et al. 2016). In patients with end-stage neurological disease, chronic activation of astrocytes by IL-1β caused downregulation of TIMP-1 mRNA and protein expression in CSF and brain tissues (Gardner and Ghorpade 2003).

In our experiments, Timp-2 and Timp-3 mRNA expression significantly increased after 6 h of exposure to 50 nM and 1 μM of the VGVAPG peptide. However, knockout of the Glb1 gene abolished the effects of 1 μM of VGVAPG peptide action. Our data would suggest that activation of Timp-2 and Timp-3 mRNA expression is mainly EBP-dependent. To date, it has been reported that increased expression of TIMP-3 enhances Fas-mediated cell death through stabilisation of the FasR as well as by preventing shedding of FasL from the extracellular matrix (Ahonen et al. 2003; Mitsiades et al. 1998). Furthermore, it has also been demonstrated that integrin α3β1 as a TIMP-2 receptor results in inhibition of fibroblast growth factor (FGF) or VEGF-induced angiogenesis by effectively blocking the association of HSP60 and SHP-1 to this receptor (Seo et al. 2003). High mRNA expression of Timp-2 and Timp-3 was detected in zones of neurogenesis in rat brains and these proteins are considered important in cellular proliferation and/or differentiation (Jaworski and Fager 2000; Vaillant et al. 1999). Furthermore, enhanced TIMP-3 mRNA expression was found in normal human proliferating keratinocytes during the wound healing process (Vaalamo et al. 1999). It has also been shown that TIMP-3 possesses growth factor-like properties and its presence is required for the growth of oligodendrocytes (Gomez et al. 1997; Oh et al. 1999).

After 3 h of exposure to 1 and 50 μM of VGVAPG peptide, Timp-4 mRNA expression significantly increased; however, after 6 h of exposure to 50 nM and 1 μM of VGVAPG peptide, Timp-4 mRNA expression decreased. Silencing the Glb1 gene
protected the cells against changes caused by 1 μM of VGVAPG peptide in Timp-4 mRNA expression after 3 h; however, after 6 h, mRNA expression of Timp-4 significantly increased. Our data suggest that Timp-4 mRNA expression can be partially EDP-dependent. TIMP-4 has been poorly studied and data concerning its expression and function are limited. To date, it has been shown that TIMP-4 expression increases during damage of blood vessels and is correlated with a developing inflammation (Koskivirta et al. 2006). Moreover, upregulation of TIMP-4 in response to inflammation in CSF from patients with tropical spastic paraparesis or myelopathy associated with T cell lymphotropic virus was observed (Kettlun et al. 2003). Research conducted on human gliomas has shown that TIMP-4 substantially reduced glioma’s invasive capacity, although without decreased cell viability and proliferation (Groft et al. 2001). Currently, it is believed that TIMP-4 mRNA and protein upregulation appear in a pathological inflammation where extracellular matrix remodeling is usually induced and in the wound repair process (Koskivirta et al. 2006; Vaalamo et al. 1999).

Interestingly, Fager and Jaworski (2000) studied mRNA expression of Timp-1, Timp-2, Timp-3 and Timp-4 during rat central nervous system development. They showed that in whole-brain extracts, Timp-1 mRNA slowly disappeared in favour of gradually increasing Timp-4. Furthermore, Timp-2 and Timp-3 mRNA slowly grew throughout the rat’s entire lifespan. The whole Timp mRNA expression pattern obtained in our study is consistent with that previously found by Fager and Jaworski (2000) during development of CNS.

### Conclusion

Our study is the first to have investigated the VGVAPG mechanism of action in mouse primary glial cells in vitro. Our experiments show that VGVAPG in a wide range of concentrations exhibits neither cytotoxic nor proapoptotic properties in mouse glial cells. Our research shows that an increase in mRNA expression of Timp-2 and Timp-3 genes in mouse glial cells in vitro is EBP-dependent. Nevertheless, changes in mRNA expression of Mmp-2, Mmp-9 and Timp-4 genes in mouse glial cells in vitro can be partially EBP-dependent. Decreased mRNA expression of Timp-1 is probably EBP-independent. Our data suggest that VGVAPG peptides sensitize mouse glial cells in vitro to apoptotic or pro-inflammatory signals from the brain microenvironment. However, we cannot exclude that the increasing expression of Timp-2, Timp-3 and Timp-4 mRNA also facilitates brain repair after a stroke by increasing cell proliferation and/or differentiation. Our research is pioneering in this research field and has a preliminary character; further studies underlying VGVAPG peptide mechanisms of action in the nervous system are thus necessary.

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### Compliance with Ethical Standards

All procedures were approved by a Bioethics Commission (no. 46/2014, First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow), as compliant with European Union law.

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