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HYPOXIC REGULATION OF THE EXPRESSION OF ANTI-ANGIOGENIC GENES IN U87 GLIOMA CELLS WITH LOSS OF FUNCTION OF ERN1 SIGNALING ENZYME

K. I. Kubaichuk[1], ***D. O. Minchenko***[1,2], ***S. V. Danilovskyi***[1], ***A. Y. Kuznetsova***[1], ***A. K. Jasim***[1], ***O. H. Minchenko***[1]

[1]*Palladin Institute of Biochemistry, NAS of Ukraine, 9, Leontovych St., Kyiv 01601, Ukraine*

[2]*O.O. Bohomoletz National Medical University, 13, Shevchenko Boulvd., Kyiv 01601, Ukraine*
e-mail: *ominchenko@yahoo.com*

The angiogenesis is an important component of tumor growth and tightly associated with hypoxia. The expression level of genes related to regulation of angiogenesis (*BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5* and *FGF2*) in glioma U87cells and cells with suppressed function of signaling enzyme ERN1, a major mediator of the endoplasmic reticulum stress by qPCR, was studied. We have shown that the expression of genes encoding BAI2, SPARC, TIMP2, TIMP3, THBS1 and THBS2 is strongly increased in glioma cells with ERN1 signaling enzyme loss of function, being more intense for *TIMP2, TIMP3* and *THBS1* genes. At the same time, the expression of genes encoding TIMP1, TIMP4, ADAMTS5 and FGF2 is significantly decreased with more strong effect for ADAMTS5 and *TIMP4* genes. At hypoxia, the expression of most of studied genes in both glioma cell types is affected. Hypoxia induced the expression of *TIMP1, TIMP3* and *ADAMTS5* genes both in control glioma cells and cells with ERN1 enzyme loss of function. However, the effect of hypoxia towards *TIMP2* gene expression was observed only in control glioma cells. At the same time, the expression of genes encoding BAI2, SPARC, THBS1, THBS2, ADAMTS5 and FGF2 is decreased under hypoxia action, but its expression mostly depended on ERN1 signaling enzyme function. The results of this study provide strong evidence that suppression of ERN1 signaling enzyme function, as well as hypoxia, affects the expression of genes related to regulation of angiogenesis in glioma cells. It is suggested that changes in the expression of these genes contribute to a suppression of glioma cells' proliferation by blockade of functioning of ERN1 signaling enzyme.

Keywords: gene expression, BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5, FGF2, ERN1, glioma cells, hypoxia.

INTRODUCTION

The endoplasmic reticulum stress plays an important role in tumor growth. It is an obligatory component of tumorigenesis, as well as hypoxia and angiogenesis. Moreover, the malignant tumors use the endoplasmic reticulum stressing for activation of the proliferative processes partially through the enhancement of an angiogenesis and suppression of apoptosis, in parallel to knockdown of the tumor suppression genes [1, 2]. Different factors, including hypoxia, have been shown to induce complex intracellular signaling events known as the unfolded protein response which is mainly mediated by endoplasmic reticulum to nuclei-1 signaling enzyme (ERN1; also named as inositol requiring enzyme-1alpha, IRE1) in order to adapt cells for survival or, alternatively, to enter cell death programs through the endoplasmic reticulum-associated machineries [3–5]. As such, it participates in the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum [2]. The endoplasmic reticulum stress is contributed to the expression profile of many regulatory genes resulting in proliferation, angiogenesis and apoptosis [6, 7].

Two distinct catalytic domains of the bifunctional signaling enzyme ERN1 were identified: a serine/threonine kinase and an endoribonuclease which contribute to ERN1 signalling. The ERN1-associated kinase activity autophosphorylates and dimerizes this enzyme, leading to the activation of its endoribonuclease domain, degradation of a specific subset of mRNA and initiation of the pre-XBP1 (X-box binding protein 1) mRNA splicing [6, 8, 9]. Mature XBP1 mRNA splice variant encodes a transcription factor that stimulates the expression of hundreds of unfolded protein response-specific genes [6, 10]. Thus, a known role of the spliced form of XBP1 in metabolic processes is its ability to act as a transcription factor regulating the expression of genes that increase the endoplasmic reticulum folding capacity, thereby improving cell surviving. Moreover, XBP1s has additional functions, especially in the regulation of glucose homeostasis [11, 12]. Thus, the p38 MAP kinase phosphorylates the spliced form of XBP1 and significantly enhances its nuclear migration [11]. Moreover, the regulatory subunits of phosphatidyl inositol 3-kinase interact with XBP1 and also increase its nuclear translocation [13]. Zhou et al. [12] shown that XBP1s interacts with the Forkhead box O1 (FOXO1) transcription factor and directs it toward the proteasome-mediated degradation.

The endoplasmic reticulum stress response-signalling pathway is associated with hypoxia and linked to the neovascularization process and tumor growth since the complete blockade of ERN1 signal transduction pathway had anti-tumor effects preferentially via a suppression of VEGFA [14, 15]. The malignant tumor angiogenesis is regulated by different, tightly interconnected factors. However, matrix proteins with pleiotropic roles which are linked to tumor growth represent special interest because of revealed anti-angiogenic properties and a capacity to regulate cell proliferation [16–22].

Thrombospondin 1 (THBS1 or TSP1) is an adhesive glycoprotein that mediates cell-to-cell interactions, has anti-angiogenic properties and inhibits of cell growth [17]. It was shown that decorin which antagonizes the angiogenic network induces the expression of thrombospondin-1 and tissue inhibitor of matrix metalloproteinase 3 (TIMP3), but inhibits vascular endothelial growth factor A (VEGFA) [16]. Moreover, the endothelial nitric oxide synthase controls the expression of the angiogenesis inhibitor thrombospondin 2 and ADAM metallopeptidase with thrombospondin type 1 motif, 5 (ADAMTS5).

There are four genes encoded tissue inhibitor of matrix metalloproteinase with different regulatory properties. TIMP1 is a potent inhibitor of tumor growth and angiogenesis, but is also able to promote cell proliferation in a wide range of cell types. TIMP2 has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells.

The enhanced neovascularization correlated with down-regulation of anti-angiogenic THBS1 and related proteins, such as connective tissue growth factor (CTGF) [21]. Moreover, VEGFA binds connective tissue growth factor and that its angiogenic activity is inhibited in the VEGFA-CTGF complex form; however, stability of this complex, as well as the angiogenic activity of VEGF depends from matrix metalloproteinases and its inhibitors [22]. The brain-specific angiogenesis inhibitor 2 (BAI2) inhibits the ischemia-induced brain angiogenesis [23].

SPARC is a pleiotropic protein, has anti-angiogenic properties and appears to regulate cell growth [24]. The endoplasmic reticulum stress is recognized as an important determinant of tumor growth and mainly contributes to the expression profile of genes controlling angiogenesis; however, a detailed molecular mechanism of the angiogenesis regulation is not yet clear and remains to be determined. It is important in studying the role of ERN1 signaling pathways in tumor progression for developing a new understanding concerning molecular mechanisms of malignant tumors progression in relation to hypoxia and defines the best targets for the design of specific inhibitors that could act as potent antitumor drugs.

The main goal of this work was to study the role of the expression of genes related to the regulation of angiogenesis (*BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5* and *FGF2*) in glioma U87 cells for evaluation of its significance in suppressing tumor growth through a blockade of the EREN1 signaling enzyme functioning.

MATERIALS AND METHODS

Cell lines and culture conditions. The glioma cell line U87 was obtained from ATCC (U.S.A.) and grown in high glucose (4,5 g/l) Dulbecco's modified Eagle's minimum essential medium (Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio,Inc., USA), penicillin (100 units/ml; Gibco) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO2 incubator. In this work we used two sublines of this glioma cell line. One subline was obtained by selection of stable transfected clones with overexpression of vector, which was used for creation of dnERN1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of hypoxia and glutamine or glucose deprivations on the expression level of genes related to regulation of angiogenesis. Second subline was obtained by selection of stable transfected clones with overexpression of ERN1 dominant/negative constructs (dnERN1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme [14]. The level of expression of studied genes in these cells was compared with such level in cells transfected by the vector (control 1). That sub-line was also used as control 2 for investigation of the effect of hypoxia and glutamine or glucose deprivations on the expression level of genes related to regulation of angiogenesis under blockade ERN1 enzyme function.

Hypoxic conditions were created in special incubator with 3% oxygen and 5% carbon dioxide levels; culture plates with complete DMEM were exposed to these conditions for 16 hrs.

The functional activity of ERN1 signaling enzyme in glioma cells that over-expresses a dominant-negative construct of endoplasmic reticulum–nuclei-1 (dnERN1) was estimated by analysis of the expression of XBP1 alternative splice variant (XBP1s), a key transcription factor in ERN1 signaling, and phosphorylated isoform of ERN1 in cells under endoplasmic reticulum stress condition induced by tunicamycin (0.01 mg/ml during 2 hrs) [25].

RNA isolation. Total RNA was extracted from different tumor tissues and normal tissue counterparts, as described previously назвати принцип методу [25]. RNA pellets were washed with 75% ethanol and dissolved in nuclease-free distilled water.

Reverse transcription and quantitative real-time polymerase chain reaction analysis. The expression levels of brain-specific angiogenesis inhibitor 2 (*BAI2*), fibroblast growth factor 2 (*FGF2*), secreted protein acidic and rich in cysteine (*SPARC*), tissue inhibitor of matrix metalloproteinase 1–4 (*TIMP1–TIMP4*), thrombospondin 1 and 2 (*THBS1 and THBS2*), ADAM metallopeptidase with thrombospondin type 1 motif, 5 (*ADAMTS5*) and secreted protein acidic and rich in cysteine (*SPARC*) mRNAs were measured in glioma cell line U87 and its subline with a deficiency of ERN1 by quantitative polymerase chain reaction of cDNA using „Mx 3000P QPCR" (Stratagene, U.S.A.) and SYBRGreen Mix (AB gene, Great Britain). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis as described previously [25]. Polymerase chain reaction was performed in triplicate. For amplification of *BAI2* cDNA was used forward (5′-CATTGTCCTGGTGAACATGC-3′ and reverse (5′-TGCACAGCAGTGATGACAAA-3′) primers**.** The nucleotide sequences of these primers correspond to sequences 3612–3631 and 3959–3940 of human *BAI2* cDNA (GenBank accession number NM_001703). The size of amplified fragment is 348 bp. The amplification of *FGF2* cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′-AGAGCGACCCTCACATCAAG-3′ and reverse – 5′-ACTGCCCAGTTCGTTTCAGT-3′. The nucleotide sequences of these primers correspond to sequences 571–590 and 804–785 of human *FGF2* cDNA (GenBank accession number NM_002006). For amplification of *TIMP1* cDNA we used forward (5′-AATTCCGACCTCGTCATCAG-3′ and reverse (5′-TGCAGTTTTCCAGCAATGAG-3′) primers. The nucleotide sequences of these primers corresponds to sequences 301–320 and 530–511 of human *TIMP1* cDNA (GenBank accession number NM_003254). The size of amplified fragment is 230 bp. The amplification of *TIMP2* cDNA was performed using forward primer (5′-GATGCACATCACCCTCTGTG-3′) and reverse primer (5′-GTCGAGAAACTCCTGCTTGG-3′). These oligonucleotides correspond to sequences 665–684 and 950–931 of human *TIMP2* cDNA (GenBank accession number NM_003255). The size of amplified fragment is 286 bp. Two other primers were used for real time RCR analysis of the expression of *TIMP3* cDNA: forward – 5′-CTGACAGGTCGCGTCTATGA-3′ and reverse – 5′-GGCGTAGTGTTTGGACTGGT-3′. The nucleotide sequences of these primers correspond to sequences 1496–1515 and 1735–1716 of human *TIMP3* cDNA (GenBank accession number NM_000362). The size of amplified fragment is 240 bp. For amplification of *TIMP4* cDNA we used forward (5′-CAGACCCTGCTGACACTGAA-3′ and reverse (5′-AGACTTTCCCTCTGCAC-

CAA-3′) primers. The nucleotide sequences of these primers correspond to sequences 696–715 and 954–935 of human *TIMP4* cDNA (GenBank accession number NM_003256). The size of amplified fragment is 259 bp. For real time RCR analysis of *THBS1* cDNA expression we used next primers: forward – 5′-TTCTACGAGCTGTGGCAATG-3′ and reverse – 5′-TTTCTTGCAGGCTTTGGTCT-3′. The nucleotide sequences of these primers correspond to sequences 1352–1371 and 1637–1618 of human *THBS1* cDNA (GenBank accession number NM_003246). The size of amplified fragment is 286 bp. The amplification of *THBS2* cDNA was performed using forward primer (5′-AGCGTCAGATGTGCAACAAG-3′) and reverse primer (5′-CTTGTCCTTGCATGGGTTTT-3′). These oligonucleotides correspond to sequences 1864–1883 and 2213–2194 of human *THBS2* cDNA (GenBank accession number NM_003247). The size of amplified fragment is 350 bp. The amplification of *ADAMTS5* cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′-GCCTGGAAGTGAGCAAGAAC-3′ and reverse – 5′-TTTCGTGAGCCACAGTGAAG-3′. The nucleotide sequences of these primers correspond to sequences 1709–1728 and 1963–1944 of human *ADAMTS5* cDNA (GenBank accession number NM_007038). The size of amplified fragment is 255 bp. The amplification of *SPARC* cDNA for qRCR analysis was performed using two oligonucleotides primers: forward – 5′-TGCCTGATGAGACAGAGGTG-3′ and reverse – 5′-AAGTGGCAGGAAGAGTCGAA-3′. The nucleotide sequences of these primers correspond to sequences 176–195 and 479–460 of human SPARC cDNA (GenBank accession number NM_003118). The size of amplified fragment is 304 bp. The amplification of *ACTB* (beta-actin) cDNA was performed using forward – 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse – 5′-AGCACTGTGTTGGCGTACAG-3′ primers. These primers nucleotide sequences correspond to 747–766 and 980–961 of human ACTB cDNA (GenBank accession number NM_001101). The size of amplified fragment is 234 bp. The expression of *ACTB* mRNA was used as control of analyzed RNA quantity. The primers were received from „Sigma" (USA). An analysis of quantitative PCR was performed using special computer program „Differential expression calculator" and statistical analysis – in Excel program. The values of *BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5* and *FGF2* mRNA expressions were normalized to the expression of beta-actin mRNA and represent as percent of control 1 (100%). All values are expressed as mean ± SEM from triplicate measurements performed in three independent experiments.

3. RESULTS

In this study, we found that genes related to the regulation of angiogenesis and proliferation are expressed in human glioma cell line U87, and the levels of their expression were dependent upon the ERN1 signaling enzyme functioning. Thus, the expression of *BAI2* gene in glioma cells deficient in signaling enzyme ERN1 is increased almost 4-fold as compared to control glioma cells (Fig. 1,*A*). An exposure of cells for 16 hrs to hypoxia did not change significantly the expression of *BAI2* gene in control glioma cells, but hypoxia inhibited its expression in cells with suppressed function of signaling enzyme ERN1 (Fig. 1, *A*). As shown in Fig. 1, *B*, the blockade of ERN1 enzyme function induced more that 4-fold the expression of *SPARC* gene. Moreover, the expression of that gene was also induced (approximately in 4-fold) by hypoxia, but only in control glioma cells.

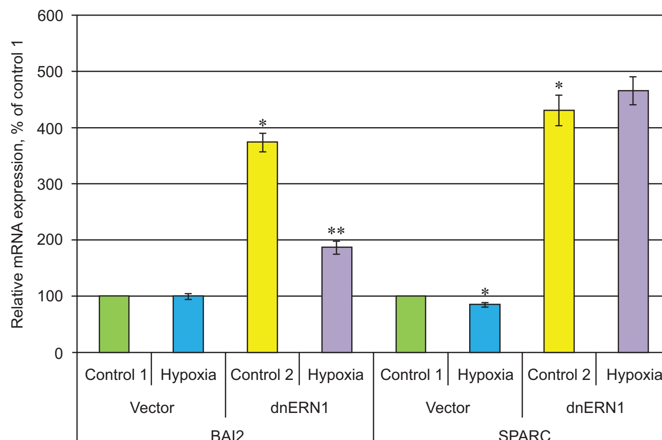

Fig. 1. Effect of hypoxia on the expression level of brain-specific angiogenesis inhibitor-2 (*BAI2*) and secreted protein acidic and rich in cysteine (*SPARC*) mRNA in glioma U87 cells (Vector) and its variant with suppressed function of ERN1 signaling enzyme (dnERN1). The values of *BAI2* and *SPARC* mRNA expressions were normalized to the expression of beta-actin mRNA and are represented as a percent of control 1. Data are expressed as mean ± SEM of values from each group; * – P < 0.05 versus control 1; ** – P < 0.05 versus control 2

Рис. 1. Ефект гіпоксії на рівень експресії мРНК інгібітора ангіогенезу-2, специфічного для мозку (BAI2), та кислого і збагаченого на цистеїн протеїну, що секретується (*SPARC*), у клітинах гліоми лінії U87 (Vector) та її варіанта з пригніченою функцією сигнального ензиму ERN1 (dnERN1). Значення експресії мРНК *BAI2* та *SPARC* нормалізували за експресією мРНК бета-актину і представляли як відсоток від контролю 1. Результати виражали середнім значенням ± SEM для кожної із груп; * – P < 0,05 порівняно з контролем 1; ** – P < 0,05 порівняно з контролем 2

At the same time, the expression of *FGF2* and *ADAMTS5* genes in glioma cells with ERN1 loss of function, as compared to control glioma cells, was strongly decreased – 3- and 11-fold, correspondingly (Fig. 2). Hypoxia decreased the expression of *FGF2* gene, but increased *ADAMTS5* gene in both types of glioma cells, and the effect of hypoxia did not depend significantly on ERN1 signaling enzyme functioning.

Analysis of the expression of tissue inhibitor of matrix metalloproteinase genes in glioma cells with suppressed function of signaling enzyme ERN1 has shown that *TIMP1* and *TIMP4* gene expression is reduced as compared to control glioma cells (Fig. 3). However, the expression of *TIMP2* and *TIMP3* genes was strongly (more than 7-fold) increased (Fig. 4). An exposure of glioma cells for 16 hrs to hypoxia led to an increase of *TIMP1* and *TIMP3* gene expressions in both types of studied glioma cells, independently on ERN1 enzyme function. However, much stronger effect of hypoxia (almost 3-fold) was shown for *TIMP3* gene (Fig. 3 and 4). At the same time, the blockade the ERN1 signaling enzyme function completely eliminated the increasing effect of hypoxia on *TIMP2* gene expression. No significant changes in the expression of *TIMP4* gene were observed in both types of used glioma cells treated by hypoxia (Fig. 4).

As shown in Fig. 5, in glioma cells with ERN1 loss of function as compared to control glioma cells the expression of *THBS1* and *THBS2* genes is strongly increased, being more pronounced for *THBS1* gene. No significant changes in the expression of *THBS1* gene were observed in control glioma cells, but blockade of ERN1 signaling enzyme function led to a decrease of expression of that gene (Fig. 5, *A*). At the same time, the expression of *THBS2* gene was decreased 2-fold in control glioma cells sub-

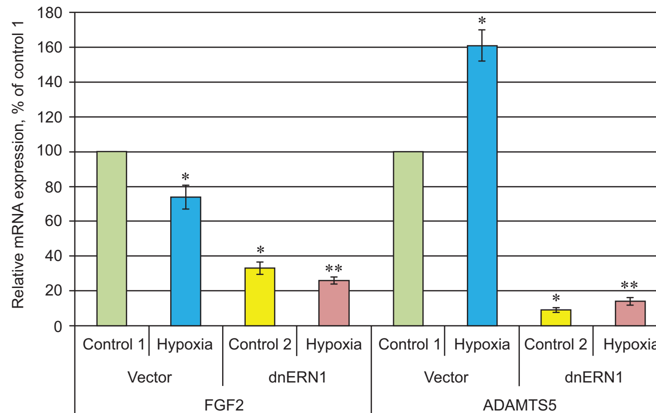

Fig. 2. Effect of hypoxia on the expression level of fibroblast growth factor 2 (*FGF2*) and ADAM metallopeptidase with thrombospondin type 1 motif, 5 (*ADAMTS5*) mRNA in glioma U87 cells (Vector) and its variant with suppressed function of ERN1 signaling enzyme (dnERN1). The values of *FGF2* and *ADAMTS5* mRNA expressions were normalized to the expression of beta-actin mRNA and are represented as a percent of control 1. Data is expressed as mean ± SEM of values from each group; * – $P < 0.05$ versus control 1; ** – $P < 0.05$ versus control 2

Рис. 2. Ефект гіпоксії на рівень експресії мРНК фактора росту фібробластів (FGF2) та ADAM металопептидази 5 з мотивом тромбоспондину типу 1 (*ADAMTS5*) у клітинах гліоми лінії U87 (Vector) та її варіанта з пригніченою функцією сигнального ензиму ERN1 (dnERN1). Значення експресії мРНК *FGF2* і *ADAMTS5* нормалізували за експресією мРНК бета-актину та представляли як відсоток від контролю 1. Результати виражали середнім значенням ± SEM для кожної із груп; * – $P < 0{,}05$ порівняно з контролем 1; ** – $P < 0{,}05$ порівняно з контролем 2

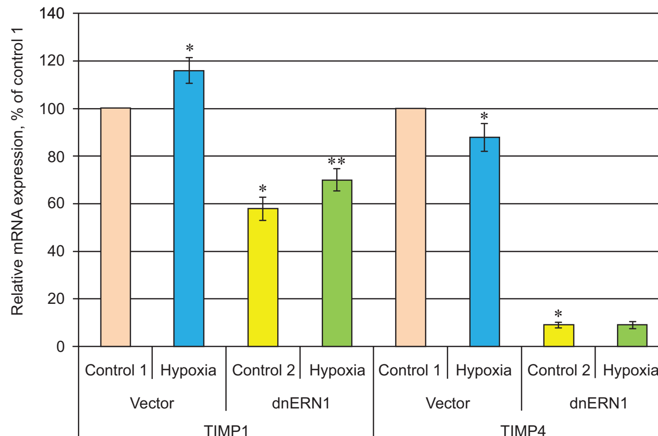

Fig. 3. Effect of hypoxia on the expression level of TIMP metallopeptidase inhibitor 1 and 4 (*TIMP1* and *TIMP4*) mRNA in glioma U87 cells (Vector) and its variant with suppressed function of ERN1 signaling enzyme (dnERN1). The values of *TIMP1* and *TIMP4* mRNA expressions were normalized to the expression of beta-actin mRNA and are represented as a percent of control 1. Data is expressed as mean ± SEM of values from each group; * – $P < 0.05$ versus control 1; ** – $P < 0.05$ versus control 2

Рис. 3. Ефект гіпоксії на рівень експресії мРНК інгібітора металопептидази TIMP 1 та 4 (*TIMP1* та *TIMP4*) у клітинах гліоми лінії U87 (Vector) та її варіанта з пригніченою функцією сигнального ензиму ERN1 (dnERN1). Значення експресії мРНК *TIMP1* та *TIMP4* нормалізували за експресією мРНК бета-актину і представляли як відсоток від контролю 1. Результати виражали середнім значенням ± SEM для кожної із груп; * – $P < 0{,}05$ порівняно з контролем 1; ** – $P < 0{,}05$ порівняно з контролем 2

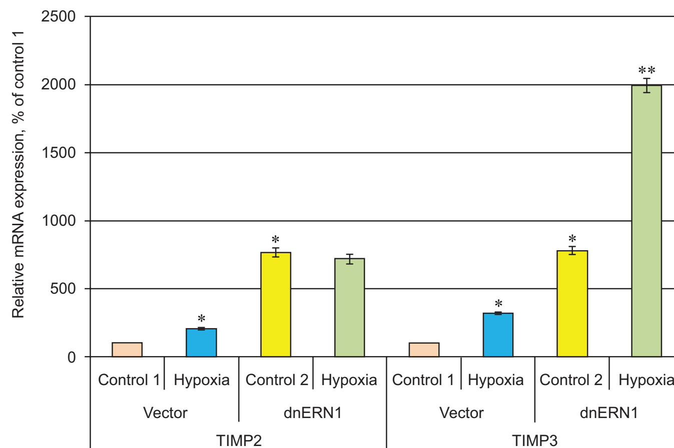

Fig. 4. Effect of hypoxia on the expression level of TIMP metallopeptidase inhibitor 2 and 3 (*TIMP2* and *TIMP3*) mRNA in glioma U87 cells (Vector) and its variant with suppressed function of ERN1 signaling enzyme (dnERN1). The values of *TIMP2* and *TIMP3* mRNA expressions were normalized to the expression of beta-actin mRNA and are represented as a percent of control 1. Data is expressed as mean ± SEM of values from each group; * – $P < 0.05$ versus control 1; ** – $P < 0.05$ versus control 2

Рис. 4. Ефект гіпоксії на рівень експресії мРНК інгібітора металопептидази TIMP 2 та 3 (*TIMP2* та *TIMP3*) у клітинах гліоми лінії U87 (Vector) та її варіанта з пригніченою функцією сигнального ензиму ERN1 (dnERN1). Значення експресії мРНК *TIMP2* та *TIMP3* нормалізували за експресією мРНК бета-актину і представляли як відсоток від контролю 1. Результати виражали середнім значенням ± SEM для кожної із груп; * – $P < 0,05$ порівняно з контролем 1; ** – $P < 0,05$ порівняно з контролем 2

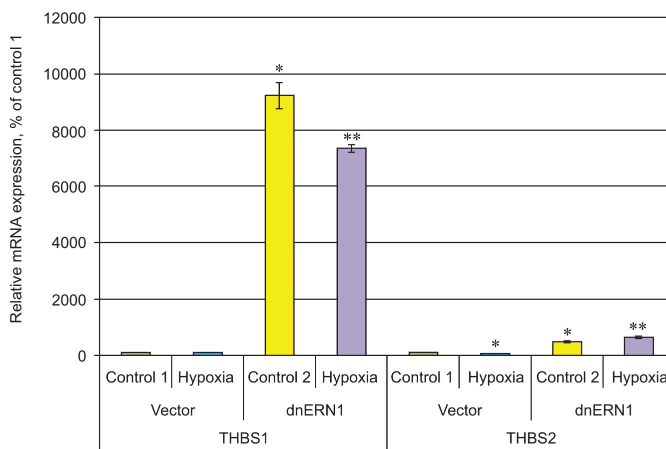

Fig. 5. Effect of hypoxia on the expression level of thrombospondin 1 and 2 (*THBS1* and *THBS2*) mRNA in glioma U87 cells (Vector) and its variant with suppressed function of ERN1 signaling enzyme (dnERN1). The values of *THBS1* and *THBS2* mRNA expressions were normalized to the expression of beta-actin mRNA and are represented as a percent of control 1. Data is expressed as mean ± SEM of values from each group; * – $P < 0.05$ versus control 1; ** – $P < 0.05$ versus control 2

Рис. 5. Ефект гіпоксії на рівень експресії мРНК тромбоспондину 1 та 2 (*THBS1* та *THBS2*) у клітинах гліоми лінії U87 (Vector) та її варіанта з пригніченою функцією сигнального ензиму ERN1 (dnERN1). Значення експресії мРНК *THBS1* та *THBS2* нормалізували за експресією мРНК бета-актину і представляли як відсоток від контролю 1. Результати виражали середнім значенням ± SEM для кожної із груп; * – $P < 0,05$ порівняно з контролем 1; ** – $P < 0,05$ порівняно з контролем 2

jected to hypoxia, but reverse changes in the expression of that gene in glioma cells with suppressed function of signaling enzyme ERN1 were observed (Fig. 5, *B*). Thus, a 2-fold increase in *THBS2* gene expression was shown in the hypoxic condition in glioma cells with ERN1 loss of function.

DISCUSSION

We have demonstrated that the expression of different genes encoding for important regulatory factors controlling angiogenesis and growth processes, is dependent from endoplasmic reticulum stress signaling mediated by ERN1 enzyme. Angiogenesis is an important component of different proliferative processes, in particular, tumor growth. We studied the expression of genes mostly related to suppression of an angiogenesis and shown that blockade of ERN1 signaling enzyme function leads to a significant increase of the expression of *BAI2, SPARC, TIMP2, TIMP3, THBS1* and *THBS2* genes in glioma cells, besides that, the most significant increase was shown for *TIMP2, TIMP3* and *THBS1* genes. These findings are largely consistent with data from previous studies about the involvement of these genes in the regulation of different proliferative processes and angiogenesis [16, 17, 21, 23, 24]. Recently, it was shown that an integrin-binding N-terminal peptide region of TIMP-2 retains potent angio-inhibitory and antitumorigenic activity *in vivo* [19]. These data together with our results can explain suppression of glioma cell proliferation by blockade the ERN1 enzyme function [14, 15]. It is possible that a significant effect in suppression of this glioma cell proliferation contributes strong induction of brain-specific angiogenesis inhibitor 2 and *SPARC* gene expression, as well as both *THBS* genes, since both of them have strong anti-angiogenic properties and regulate cell growth [23, 24].

It should be noted that angiogenesis, like many other biological processes, is regulated by complex network of different factors which are tightly interconnected [16, 18, 20, 21, 23, 25]. Thus, thrombospondin-1, a matrix-bound adhesive glycoprotein, has been shown to modulate tumor progression and up-regulates tissue inhibitor of metalloproteinase-1 production in human tumor cells and also up-regulates matrix metalloproteinases MMP-2 and MMP-9 [18, 20]. These data suggest that the balance between matrix metalloproteinases and tissue inhibitors of metalloproteinases is a key determinant in different biological effects of THBS1, including tumor cell invasion, and may provide an explanation for the divergent activities reported for thrombospondin-1 in tumor progression.

Thus, the THBS1 is involved in affecting critical balance between MMPs and their inhibitors, maintaining the controlled degradation of the extracellular matrix needed to support tumor growth and metastasis in glioma cells where we find the overexpression of THBS1 as well as THBS2, TIMPs and other factors [18–22]. TIMP-2 is involved in binding to the receptor integrin $\alpha3\beta1$ and mediates angio-inhibitory and tumor suppressor activity [19]. Moreover, the enhanced neovascularization correlated with down-regulation of anti-angiogenic connective tissue growth factor which binds VEGFA, and its angiogenic activity is inhibited in the VEGFA-CTGF complex form [22]. At the same time, stability of that complex, as well as the angiogenic activity of VEGF, depends on the matrix metalloproteinases and its inhibitors [21, 22].

It is possible that a significant reduction in the expression of *ADAMTS5, FGF2 and TIMP4* showing pro-angiogenic properties also contributed to a suppression of prolifera-

tion of the glioma cell with suppressed function of ERN1 signaling enzyme. Thus, the balance between different regulatory factors which participate in the control of angiogenesis, including vascular endothelial growth factor, a key pro-angiogenic factor, really determinate an angiogenesis in malignant tumors. As shown in Fig. 6, the regulation of angiogenesis by different factors (BAI2, FGF2, SPARC, ADAMTS5, TIMP1-4, THBS1, THBS2, endoplasmic reticulum stress and hypoxia) is complex and interconnected.

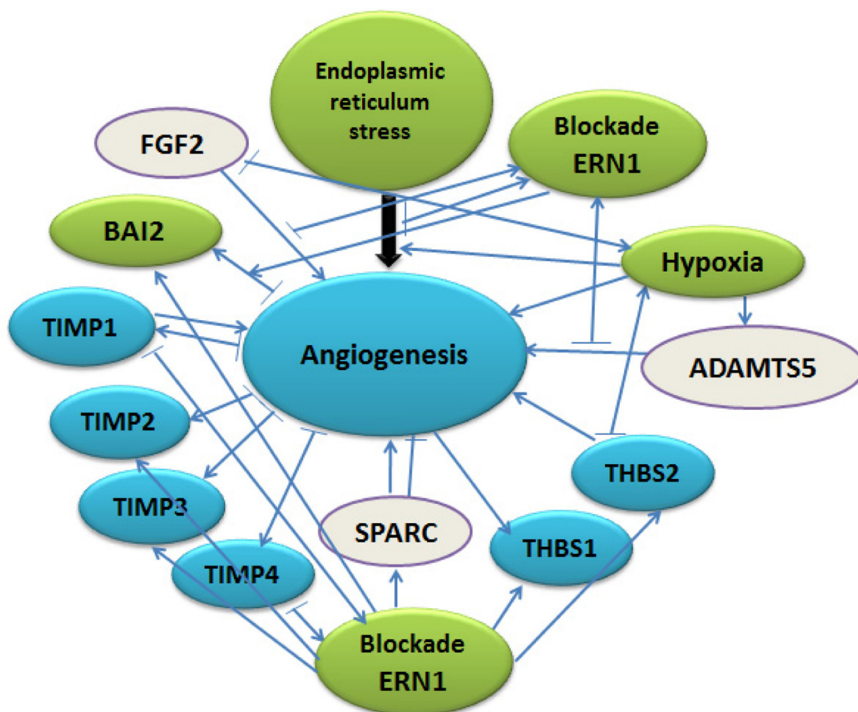

Fig. 6. Putative scheme of angiogenesis regulation by different factors, including BAI2, FGF2, SPARC, ADAMTS5, TIMP1-4, THBS1 and THBS2. Pleiotropic functions of TIMP1, TIMP4 and SPARC as well as the role of endoplasmic reticulum stress and function of ERN1, the main sensor and signaling enzyme mediated this stress, in the angiogenesis are shown

Рис. 6. Імовірна схема регуляції ангіогенезу різними факторами, включаючи BAI2, FGF2, SPARC, ADAMTS5, TIMP1-4, THBS1 і THBS2. Показана плейотропна функція TIMP1, TIMP4 та SPARC, а також роль стресу ендоплазматичного ретикулуму та функції ERN1, основного сенсорно-сигнального ензиму, що опосередковує цей стрес, у ангіогенезі

Moreover, some of these factors possess pleiotropic functions. Most of these regulatory factors participated in the endoplasmic reticulum stress and its expression in normal and hypoxic conditions mainly depends on ERN1 signaling enzyme functioning. However, the precise molecular regulatory mechanisms of angiogenesis regulation remain unclear and require additional study.

CONCLUSIONS

The results of this study provide strong evidence that the expression of genes encoding key regulatory factors related to the control of proliferation and angiogenesis in glioma cells with suppressed function of signaling enzyme ERN1 is deregulated. It is possible that changes in the expression of *BAI2, SPARC, TIMPs, THBSs, ADAMTS5* and *FGF2* genes in glioma cells can contribute to a suppression of tumor growth through modulation the angiogenesis, as well as proliferation. Thus, the obtained results underscore a crucial role of BAI2, SPARC, THBSs, TIMPs, ADAMTS5 and FGF2 in tumor progression and should be important for developing a new understanding concerning molecular mechanisms of malignant tumors growth in relation to the ERN1 signaling and hypoxia. They can also suggest the best targets for the design of specific inhibitors acting as potent antitumor drugs.

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РЕГУЛЯЦІЯ ЕКСПРЕСІЇ АНТИАНГІОГЕННИХ ГЕНІВ У КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87 З ВТРАЧЕНОЮ ФУНКЦІЄЮ СИГНАЛЬНОГО ЕНЗИМУ ERN1 ЗА УМОВ ГІПОКСІЇ

***К. І. Кубайчук*[1], *Д. О. Мінченко*[1,2], *С. В. Даніловський*[1], *А. Ю. Кузнєцова*[1], *А. К. Джассім*[1], *О. Г. Мінченко*[1]**

[1]*Інститут біохімії ім. О. В. Палладіна НАН України, вул. Леонтовича, 9, Київ 01601, Україна*

[2]*Національний медичний університет ім. О. О. Богомольця, бульв. Шевченка, 13, Київ 01601, Україна*

e-mail: ominchenko@yahoo.com

Ангіогенез є важливим компонентом росту злоякісних пухлин і тісно пов'язаний з гіпоксією. Проведено дослідження рівня експресії генів, що мають відношення до ангіогенезу (*BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5* та *FGF2*) у клітинах гліоми лінії U87 та клітинах із пригніченою функцією сигнального ензиму ERN1, основного медіатора стресу ендоплазматичного ретикулуму, за допомогою кількісної ПЛР. Показано, що експресія генів, які кодують синтез BAI2, SPARC, TIMP2, TIMP3, THBS1 та THBS2, суттєво збільшується у клітинах гліоми

з пригніченою функцією сигнального ензиму ERN1, причому більш виражені зміни виявлені для генів *TIMP2, TIMP3* та *THBS1*. У той же час, експресія генів, що кодують синтез TIMP1, TIMP4, ADAMTS5 та FGF2, суттєво знижується, але більш виражено – генів ADAMTS5 та *TIMP4*. Гіпоксія порушує експресію більшості досліджених генів в обох типах клітин гліоми. Так, гіпоксія індукує експресію генів *TIMP1, TIMP3* та *ADAMTS5* як у контрольних клітинах гліоми, так і у клітинах із пригніченою функцією ензиму ERN1, хоча ефект гіпоксії на експресію гена *TIMP2* спостерігався лише у контрольних клітинах гліоми. У той же час експресія генів, що кодують синтез BAI2, SPARC, THBS1, THBS2, ADAMTS5 та FGF2, за гіпоксії знижується, але їх експресія переважно залежала від функції сигнального ензиму ERN1. Результати цього дослідження є суттєвим доказом того, що пригнічення функції сигнального ензиму ERN1, як і гіпоксія, змінюють експресію генів, що мають відношення до регуляції ангіогенезу у клітинах гліоми. Імовірно, що зміни в експресії цих генів роблять вклад у пригнічення проліферації клітин гліоми за умов блокади функції сигнального ензиму ERN1.

***Ключові слова**:* експресія мРНК, гіпоксія, експресія мРНК, BAI2, SPARC, FGF2, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5, клітини гліоми U87.

РЕГУЛЯЦИЯ ЭКСПРЕССИИ АНТИАНГИОГЕННЫХ ГЕНОВ В КЛЕТКАХ ГЛИОМЫ ЛИНИИ U87 БЕЗ ФУНКЦИИ СИГНАЛЬНОГО ЭНЗИМА ERN1 ПРИ ГИПОКСИИ

Е. И. Кубайчук[1], ***Д. А. Минченко***[1,2], ***С. В. Даниловский***[1], ***А. Ю. Кузнєцова***[1], ***А. К. Джассим***[1], ***А. Г. Минченко***[1]

[1]*Институт биохимии им. А.В. Палладина НАН Украины*
ул. Леонтовича, 9, Киев 01601, Украина

[2]*Национальный медицинский университет им. А. А. Богомольца,*
бульв. Шевченко, 13, Киев 01601, Украина
e-mail: ominchenko@yahoo.com

Ангиогенез является важным компонентом роста злокачественных опухолей и тесно связан с гипоксией. Проведено исследование уровня экспрессии генов, которые имеют отношение к ангиогенезу (*BAI2, SPARC, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5* и *FGF2*) в клетках глиомы линии U87 и клетках с угнетенной функцией сигнального энзима ERN1, основного медиатора стресса эндоплазматического ретикулума, с помощью количественной ПЦР. Показано, что экспрессия генов, которые кодируют синтез BAI2, SPARC, TIMP2, TIMP3, THBS1 и THBS2, существенно увеличивается в клетках глиомы с угнетенной функцией сигнального энзима ERN1, причем более выраженные изменения выявлены для генов *TIMP2, TIMP3* и *THBS1*. В то же время экспрессия генов, которые кодируют синтез TIMP1, TIMP4, ADAMTS5 и FGF2, существенно снижается, но более выражено – генов ADAMTS5 и *TIMP4*. Гипоксия нарушает экспрессию большинства исследованных генов в обоих типах клеток глиомы. Так, гипоксия индуцирует экспрессию генов *TIMP1, TIMP3* и *ADAMTS5* как в контрольных клетках глиомы, так

и в клетках с угнетенной функцией энзима ERN1, хотя эффект гипоксии на экспрессию гена *TIMP2* наблюдался лишь в контрольных клетках глиомы. В то же время экспрессия генов, которые кодируют синтез BAI2, SPARC, THBS1, THBS2, ADAMTS5 и FGF2, при гипоксии снижается, но их экспрессия преимущественно зависела от функции сигнального энзима ERN1. Результаты этого исследования являются существенным доказательством того, что угнетение функции сигнального энзима ERN1, как и гипоксия, изменяют экспрессию генов, которые имеют отношение к регуляции ангиогенеза в клетках глиомы. Вполне возможно, что изменения в экспрессии этих генов вносят вклад в угнетение пролиферации клеток глиомы при блокаде функции сигнального энзима ERN1.

Ключевые слова: гипоксия, экспрессия мРНК, BAI2, SPARC, FGF2, TIMP1, TIMP2, TIMP3, TIMP4, THBS1, THBS2, ADAMTS5, клетки глиомы U87.

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