ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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INHIBITION OF IRE1 MODIFIES EFFECT OF GLUCOSE DEPRIVATION ON THE EXPRESSION OF TNFα-RELATED GENES IN U87 GLIOMA CELLS

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Inhibition of IRE1 (inositol requiring enzyme-1), the major signaling pathway of endoplasmic reticulum stress, significantly decreases glioma cell proliferation and tumor growth. We have studied the expression of TNFα-related genes and effect of glucose deprivation on these gene expressions in U87 glioma cells overexpressing dominant-negative IRE1 defective in both kinase and endonuclease (dn-IRE1) activity of IRE1 with hopes of elucidating its contribution to IRE1 mediated glioma growth. We have demonstrated that glucose deprivation condition leads to down-regulation of the expression of TNFRSF11B, TNFRSF1A, TNFRSF10D/TRAILR4, and LITAF genes and up-regulation of TNFRSF10B/TRAILR2/DR5 gene at the mRNA level in control glioma cells. At the same time, the expression of TNFRSF21/DR6, TNFAIP1, TNFAIP3, TRADD, and CD70/TNFSF7 genes in control glioma cells is resistant to glucose deprivation condition. The inhibition of IRE1 modifies the effect of glucose deprivation on LITAF, TNFRSF21, TNFRSF11B, and TRADD gene expressions and induces sensitivity to glucose deprivation condition the expression of TNFRSF10B, TNFRSF1A, and CD70 genes. We have also demonstrated that the expression of all studied genes is affected in glioma cells by inhibition of IRE1, except TNFRSF1A gene, as compared to control glioma cells. Moreover, the changes in the expression of TNFRSF1A, TNFRSF10D/TRAILR4, and LITAF genes induced by glucose deprivation condition have opposite orientation to that induced by inhibition of IRE1. The present study demonstrates that fine-tuning of the expression of TNFα-induced proteins and TNF receptor superfamily genes, which related to cell death and proliferation, is regulated by IRE1, an effector of endoplasmic reticulum stress, as well as depends on glucose deprivation in gene specific manner. Thus, the inhibition of kinase and endoribonuclease activity of IRE1 correlates with deregulation of TNFα-induced protein genes and TNF receptor superfamily genes in gene specific manner and thus slower the tumor growth.

Key words: mRNA expression, endoplasmic reticulum stress, LITAF, TNFAIP1, TNFAIP3, TNFRSF21/DR6, TNFRSF10B/DR5, TNFRSF10D, TNFRSF11B, TNFRSF1A, TRADD, IRE1 inhibition, glioma cells, glucose deprivation.

The endoplasmic reticulum (ER) is the primary organelle able to activate a distinct cellular stress response, termed the Unfolded Protein Response (UPR), in which a moiety of factors (typically aggregates of misfolded proteins) triggers activation of a complex set of signaling pathways to execute a resolution to the causative stress. Malignant tumors utilize the endoplasmic reticulum stress response to adapt to stressful, environmental conditions [1-3]. Moreover, the circadian rhythms in cancer are controlled by endoplasmic reticulum-mediated mechanisms [4]. The rapid growth of solid tumors generates micro-environmental changes in association to hypoxia, nutrient deprivation and acidosis, which induce formation of new blood vessels and cell proliferation and surviving [2, 3, 5]. Those processes rely on the activation of endoplasmic reticulum stress signalling pathways [2, 3]. UPR is

mediated by three interconnected, endoplasmic reticulum-resident sensors. IRE1 (inositol requiring enzyme-1) is the most evolutionary conserved sensor that responds to protein misfolding with a highly tuned program aimed either to resolve the stress or direct the cell towards apoptosis in the case rectification is not viable, thus making it a key regulator of life and death processes [1, 6-9].

The IRE1 enzyme contains two distinct catalytic domains: protein kinase and endoribonuclease. Kinase activity is responsible for autophosphorylation of IRE1 and controls the expression of some genes [10]. Endoribonuclease activity is involved in the degradation of a specific subset of mRNA targeted to the ER to lessen the load of protein synthesis on the already stressed ER [11-13]. Endonuclease activity also initiates the cytosolic splicing of the pre-XBP1 (X-box binding protein 1) mRNA, whose mature transcript encodes for a transcription factor that stimulates the expression of numerous UPR-specific genes, namely other key transcription factors [14, 15]. Moreover, activation of the IRE1 branch of the endoplasmic reticulum stress response is intimately linked to apoptosis. The ablation of this sensor's function by a dominant-negative construct of IRE1 (dn-IRE1) has been shown to result in a significant anti-proliferative effect in glioma growth [2, 10, 16]. This is due to down-regulation of prevalent pro-angiogenic factors and up-regulation of anti-angiogenic genes, both *in vitro* and in the CAM (chorio-allantoic membrane) model, as well as in mice engrafted intracerebrally with U87 glioma cell clones [10, 17, 18]. However, the executive mechanisms of the exhibited anti-proliferative effects are not yet known. It is possible that anti-proliferative effect is also realized through mediation by tumor necrosis factor (TNF) and its receptors signaling, which are integrated into the UPR signaling pathways, to regulate cell apoptosis and proliferation [19-23]. Possible involvement of tumor necrosis factor receptor superfamily (TNFRSF) proteins such as TNFRSF21/DR6, TNFRSF10B/DR5/TRAILR2, TNFRSF10D/TRAILR4, TNFRSF11B, and TNFRSF1A as well as TNFα-induced proteins was made evidently pertinent through transcriptomic analysis of U87 glioma cells expressing the dominant-negative mutant of IRE1 [18].

The TRAIL (TNF-related apoptosis-inducing ligand) and TRAIL receptors are members of a subset of the TNF receptor superfamily known as death receptors (DR). The interaction between the TRAIL and TRAIL receptors (TRAILR1 or TRAILR2) initiates the extrinsic apoptotic pathway characterized by the recruitment of death domains, assembly of the death-inducing signaling complex (DISC), caspase activation and ultimately apoptosis. Conversely the decoy receptors TRAILR3 and TRAILR4, which lack the pro-apoptotic death domain, function to dampen the apoptotic response by competing for TRAIL ligand. TRAILR4 does not induce apoptosis, and has been shown to play an inhibitory role in TRAIL-induced cell apoptosis [20, 21, 24].

To date, the overwhelming majority of studies on TRAIL receptors have explored the role of these receptors as initiators of apoptosis. However, sporadic reports also suggest that engagement of the TRAIL receptors can lead to other outcomes such as cytokine and chemokine production, cell proliferation, cell migration and differentiation. Indeed, although transformed cells frequently express TRAIL, most do not undergo apoptosis upon engagement of these receptors and significant effort has been devoted toward exploring how to sensitize such cells to the pro-apoptotic effects of death receptor stimulation [25-29]. Moreover, the expression of TRAIL receptors is greatly elevated in many cancer types such as hepatocellular carcinoma, renal carcinoma and ovarian cancer, suggesting that such tumors benefit from the expression of these receptors [25, 6]. It is becoming increasingly clear that death receptor engagement, especially in the context of cancer, can lead to outcomes, other than apoptosis, that become subverted by certain tumors to their benefit [23, 25, 30].

It is shown that TNFRSF21/DR6 has induced apoptosis through a new pathway that is different from the type I and type II pathways through interacting with Bax protein [19]. B-cells lacking TNFRSF21 show increased proliferation, cell division and cell survival upon mitogenic stimulation (anti-CD40 and LPS); however, this gene is highly expressed in many tumor cell lines and tumor samples [25, 26]. Thus, the role of TNFRSF21/DR6 as an apoptosis-inducing receptor is less clear and perhaps cell type dependent. Nuclear death receptor TNFRSF10B/DR5/TRAILR2 inhibits maturation of the microRNA let-7 and increases proliferation of pancreatic cancer cell lines and other tumor cells [22]. Moreover, pancreatic tumor samples have increased levels of nuclear TRAILR2, which correlate with poor outcome of patients. Recently, it was shown that TNFRSF10B expression was essential

for endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells [20]. TNF receptor signaling is controlled by a set of adaptor molecules and has significant potential to exert pro-survival and protective roles in several diseases [31]. The mechanism of these receptors signal transduction is poorly understood. It was shown that mitochondrial aminopeptidase P3 (APP3m) as a new member of the TNF-TNFR2 signaling complex exerts an anti-apoptotic function and induces activation of JNK1 and JNK2 [31].

The adaptor protein TRADD (TNFRSF1A-associated via death domain) contains a death domain and interacts with TNFRSF1A. Overexpression of TRADD leads to two major TNF-induced responses: programmed cell death signaling and activation of NF-κB. It was shown that knockdown of TRADD gene expression by an antisense oligonucleotide resulted in a decrease of TRADD protein by 60%, coinciding with the increase of apoptotic cell death of up to 30% [32]. Moreover, microRNA-30c-2-3p down-regulates of TRADD and CCNE1 in breast cancer and leads to negative regulation of NF-κB signaling as well as cell cycle progression [33]. Great significance in TNFα signaling belongs to cytokine TNFSF7/CD70, a ligand for TNFRSF27/CD27, which can specifically activate TRAIL receptors and induce proliferation [36, 37]. Its expression is significantly increased in renal cell carcinoma relative to normal kidney tissue [38].

Biological effects of TNFα are realized by induction of specific genes through TNF receptors. The TNFα-induced proteins such as TNFAIP1 and TNFAIP3, which have different biological activities, are among them. Thus, miRNA-372, which plays crucial roles in gastric tumorigenesis by targeting the mRNA of TNFAIP1, an immediate-early response gene of the endothelium induced by TNFα, is up-regulated in gastric adenocarcinoma tissue and gastric carcinoma cell lines when compared to normal gastric tissues [39]. The overexpression of miR-373 in the gastric cancer cells increased cell proliferation and reduced the levels of TNFAIP1 mRNA and protein levels [39]. Moreover, a tumor suppressor RhoB interacts with TNFAIP1 to regulate apoptosis in HeLa cells via a SAPK/JNK-mediated signal transduction mechanism [40]. At the same time, TNFAIP3 is a zinc finger protein and ubiquitin-editing enzyme, and has been shown to inhibit NF-κB activation as well as TNF-mediated apoptosis and its expression is increased in a number of solid human tumors [41]. At the same time, TNFAIP3 itself is also a NF-κB dependent gene, that has also been shown to exert cell-type specific anti- or pro-apoptotic functions. Moreover, TNFAIP3 has both ubiquitin ligase and deubiquitinase activities, suppresses TP53 protein levels and is involved in the cytokine-mediated immune and inflammatory responses [42].

The LITAF (lipopolysaccharide-induced TNFα factor), which is also known as SIMPLE (small integral membrane protein of lysosome/late endosome) and PIG7 (p53-induced gene 7 protein), is an important factor in the regulation of the TNFα expression by direct binding to the promoter region of this gene. Its altered expression is associated with cancer and obesity [34, 35, 44]. The transcription of *LITAF* gene is induced by tumor suppressor p53 and has been implicated in the TP53-induced apoptotic pathway [45].

The aim of this study was to investigate the effects of glucose deprivation on the expression of genes encoding for tumor necrosis factor receptor superfamily and TNFα induced proteins, which participate in the regulation of apoptosis and cell proliferation, in glioma cells, as well as the contribution of endoplasmic reticulum stress sensor IRE1 to fine tune their expression with hopes of elucidating its role in the progression of certain cancers.

Materials and Methods

Cell lines and culture conditions. The glioma cell line U87 (HTB-14) was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37 °C in a 5% CO_2 incubator.

In this work we used sublines of U87 glioma cells, which were described previously [10, 18, 46]. One subline was obtained by selection of stable transfected clones overexpressing vector (pcDNA3.1), which was used for creation of dominant-negative constructs of IRE1 (dn-IRE1). This untreated subline of glioma cells was used as a control (control glioma cells) in the study of the effects of inhibition of IRE1, in regards to the expression of the TNF receptor superfamily members and TNFα-induced proteins of interest. The second sub-line was obtained by the selection of stable transfected clones overexpressing dn-IRE1, having suppression

of both the protein kinase and endoribonuclease activities of IRE1 [18]. It has recently been shown that these cells have a low rate of proliferation and do not express spliced XBP1, a key transcription factor in IRE1 signaling, after induction of endoplasmic reticulum stress by tunicamycin [18]. The expression of the studied genes was compared with cells transfected with the previously mentioned, empty vector (control glioma cells, pcDNA3.1).

Reverse transcription and qPCR analysis. QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to manufacturer's protocol. The expression level of TNFRSF21, TNFRSF10B, TNFRSF10D, TNFRSF11B, TNFRSF1A, LITAF/PIG7, TNFAIP1, TNFAIP3, TNFSF7/CD70, TRADD, and ACTB mRNA were measured in U87 glioma cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich, USA.

RNA isolation. Total RNA was extracted from glioma cells using Trizol reagent according to manufacturer's protocols (Invitrogen, USA) as described previously [10]. The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95 % ethanol and re-dissolved again in nuclease-free water.

Reverse transcription and quantitative PCR analysis. QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to manufacturer's protocol. The expression level of TNFRSF21, TNFRSF10B, TNFRSF10D, TNFRSF11B, TNFRSF1A, LITAF/PIG7, TNFAIP1, TNFAIP3, TNFSF7/CD70, TRADD, and ACTB mRNA were measured in U87 glioma cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich, USA.

For amplification of TNFRSF21 (tumor necrosis factor receptor superfamily, member 21), also known as death receptor 6 (DR6), cDNA we used next forward and reverse primers: 5′–TGATTGTGCTTTTCCTGCTG–3′ and (5′–CTCACTGGCATTGCAAAGAA–3′, correspondingly. The nucleotide sequences of these primers correspond to sequences 1491–1510 and 1759–1740 of human TNFRSF21 cDNA (GenBank accession number NM_0014452). The size of amplified fragment is 250 bp.

The amplification of TNFRSF10B (tumor necrosis factor receptor superfamily, member 10B), also known as TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2) and death receptor 5 (DR5), cDNA was performed using forward primer (5′–TGCAGCCGTAGTCTTGATTG–3′) and reverse primer (5′–TCCTGGACTTCCATTTCCTG–3′). These oligonucleotides correspond to sequences 953–972 and 1171–1152 of human TNFRSF10B cDNA (GenBank accession number NM_003842). The size of amplified fragment is 219 bp.

The amplification of TNFRSF10D (tumor necrosis factor receptor superfamily, member 10D), also known as TNF-related apoptosis-inducing ligand receptor 4 (TRAILR4), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′–CAGGAAATCCAAGGTCAGGA–3′ and reverse – 5′– AGCCTGCCTCATCTTCTTCA–3′. The nucleotide sequences of these primers correspond to sequences 969–988 and 1233–1214 of human TNFRSF10D cDNA (GenBank accession number NM_003840). The size of amplified fragment is 265 bp.

For amplification of TNFRSF11B (tumor necrosis factor receptor superfamily, member 101B) cDNA we used next forward and reverse primers: 5′–TGCAGTGTCTTTGGTCTCCT–3′ and 5′– TTCTTGTGAGCTGTGTTGCC–3′, correspondingly. The nucleotide sequences of these primers correspond to sequences 819–838 and 1067–1048 of human TNFRSF11B cDNA (GenBank accession number NM_002546). The size of amplified fragment is 249 bp.

The amplification of TNFRSF1A (tumor necrosis factor receptor superfamily, member 1A) cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′–TGTGCCTACCCCAGATTGAG–3′ and reverse – 5′–GACTGAAGCTTGGGTTTGGG–3′. The nucleotide sequences of these primers correspond to sequences 884–903 and 1126–1107 of human TNFRSF1A cDNA (GenBank accession number NM_001065). The size of amplified fragment is 243 bp.

For amplification of TRADD (TNFRSF1A-associated via death domain) cDNA we used forward (5′–TGCAGATGCTGAAGATCCAC–3′ and reverse (5′–GCTCAGCCAGTTCTTCATCC–3′)

primers. The nucleotide sequences of these primers correspond to sequences 256–275 and 543–524 of human TRADD cDNA (GenBank accession number NM_003789). The size of amplified fragment is 288 bp.

The amplification of TNFSF7 (tumor necrosis factor ligand superfamily, member 7), also known as CD70 molecule (CD70), cDNA was performed using forward primer (5′–CTGCTTTGGTCCCATTG-GTC–3′) and reverse primer (5′–CAGTATAGC-CTGGGGTCCTG–3′). These oligonucleotides correspond to sequences 212–231 and 374–355 of human TNFRSF10 cDNA (GenBank accession number NM_001252). The size of amplified fragment is 163 bp.

The amplification of TNFAIP1 (tumor necrosis factor α-induced protein 1, endothelial) cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′–GGCTG-GTGAATATGTGCCAG–3′ and reverse – 5′–GG-GAGAGCTTGTCAAACAGC–3′. The nucleotide sequences of these primers correspond to sequences 793–812 and 1020–1001 of human TNFAIP1 cDNA (GenBank accession number NM_021137). The size of amplified fragment is 228 bp.

For amplification of TNFAIP3 (tumor necrosis factor α-induced protein 3) cDNA we used next forward and reverse primers: 5′–CAAGGAAA-CAGACACACGCA–3′ and 5′–AAAGGGGC-GAAATTGGAACC–3′, correspondingly. The nucleotide sequences of these primers correspond to sequences 712–731 and 1002–983 of human TNFAIP3 cDNA (GenBank accession number NM_006290). The size of amplified fragment is 291 bp.

The amplification of LITAF (lipopolysaccharide-induced TNF factor), also known as PIG7 (p53-induced gene 7 protein), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5′–GATCGTGAGTCAGCT-GTCCT–3′ and reverse – 5′–TGAAGCTGGATGA-GAGGTGG–3′. The nucleotide sequences of these primers correspond to sequences 539–558 and 785–766 of human LITAF cDNA (GenBank accession number NM_004862). The size of amplified fragment is 247 bp.

The amplification of β-actin (ACTB) cDNA was performed using forward – 5′–GGACTTCGAG-CAAGAGATGG–3′ and reverse – 5′–AGCACTGT-GTTGGCGTACAG–3′ primers. These primer 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 beta-actin mRNA was used as control of analyzed RNA quantity.

An analysis of quantitative PCR was performed using special computer program Differential Expression Calculator. The values of the expression of TNFRSF21, TNFRSF10B, TNFRSF10D, TNFRSF11B, TNFRSF1A, LITAF/PIG7, TNFAIP1, TNFAIP3, TNFSF7/CD70, TRADD and ACTB mRNA were normalized to β-actin mRNA expressions and represented as percent of control (100%).

Statistical analysis. Statistical analysis was performed according to Student's *t*-test using Excel program as described previously [47]. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments.

Results and Discussion

To determine if glucose deprivation affects the expression of a subset of genes encoding for tumor necrosis factor-related proteins through the IRE1 branch of endoplasmic reticulum stress response, we investigated the effect of glucose deprivation condition on mRNA expression levels of different members of TNF receptor superfamily and TNFα induced proteins as well as LITAF/PIG7, which can mediate the TNFα expression and implicated in the TNF-induced apoptotic pathway. As shown in Fig. 1, *A*, the exposure of control glioma cells (transfected by empty vector pcDNA3.1) upon glucose deprivation condition does not change significantly the expression level of TNFRSF21/DR6 mRNA, but the inhibition of IRE1 induces the sensitivity of this gene expression to glucose deprivation: the level of TNFRSF21/DR6 mRNA expression is 1.8-fold decreased. Moreover, the expression level of this mRNA strongly increased (7.5-fold) in glioma cells without IRE1 signaling enzyme function as compared to control glioma cells (Fig. 1, *A*). At the same time, the expression of gene encoding for TNFRSF10B/DR5, which is also known as TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2) is slightly increased in control glioma cells upon glucose deprivation condition; however, the inhibition of IRE1 significantly enhances the effect of glucose deprivation on this gene expression (39% upregulation) (Fig. 1, *B*). Furthermore, in glioma cells without IRE1 signaling enzyme function the expression level of TNFRSF10B mRNA is strongly downregulated (2.3-fold) as compared to control glioma cells (Fig. 1, *B*).

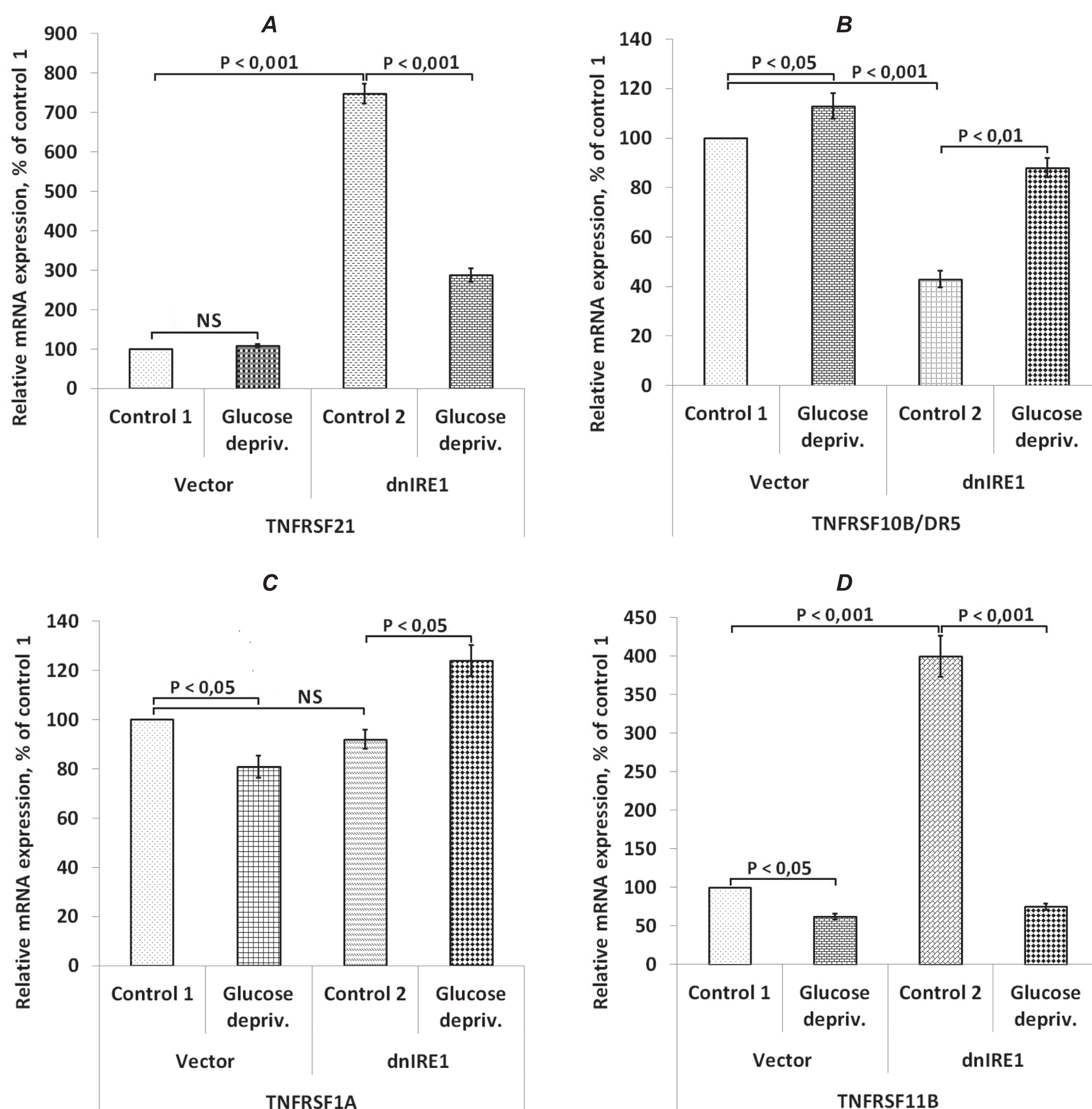

Fig. 1. Effect of glucose deprivation condition on the expression levels of TNFRSF21 (A), TNFRSF10B (B), TNFRSF1A (C), and TNFRSF11B (D) mRNAs (by qPCR) in glioma cells with a deficiency of IRE1 (dn-IRE1). These mRNA expressions values were normalized to β-actin mRNA expression and represented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4

As shown in Fig. 1, *C*, glucose deprivation down-regulates (-19%) the expression level of *TNFRSF1A* gene in control glioma cells and up-regulates (+34%) in cells overexpressed dn-IRE1 (without IRE1 signaling enzyme function). Moreover, the inhibition of IRE1 in glioma cells does not change significantly the expression of this mRNA as compared to control cells (Fig. 1, *C*). We also studied the effect of glucose deprivation condition on the expression of TNFRSF11B mRNA in control glioma cells and cells without IRE1 activity. As shown in Fig. 1, *D*, glucose deprivation down-regulates the expression level of *TNFRSF10B* gene both in control glioma cells and cells overexpressed dn-IRE1: -38 % in control glioma cells and more significantly (5.3-fold) in glioma cells without IRE1 activity. At

the same time, the inhibition of IRE1 in glioma cells strongly induces the expression of TNFRSF10B mRNA as compared to control cells (Fig. 1, *D*).

The expression level of another TNF-related apoptosis-inducing ligand receptor, TRAILR2, encoded by *TNFRSF10D* gene, is changed in control glioma cells in reverse direction upon glucose deprivation condition as compared to TRAILR2 (TNFRSF10B) (Fig. 2, *A* and 1, *B*). Thus, glucose deprivation leads to down-regulation of the expression of *TNFRSF10D* gene in control glioma cells (-32%), but inhibition of IRE1 signaling enzyme eliminates this effect of glucose deprivation. However, in glioma cells with IRE1 knockdown the expression level of TNFRSF10D mRNA is strongly up-regulated (4-fold) as compared to control cells (Fig. 2, *A*). We also investigated the sensitivity to glucose deprivation of the expression of TRADD, an adaptor protein that interacts with TNFRSF1A and mediates programmed cell death signaling, and shown that in control glioma cells this gene expression is resistant to glucose deprivation condition, but inhibition of IRE1 enzyme introduces sensitivity of *TRADD* gene expression to this experimental condition: there is 46% up-regulation in cells with dn-IRE1 (Fig. 2, *B*). At the same time, the expression level of *TRADD* gene is strongly suppressed (2.5-fold) in glioma cells without IRE1 signaling enzyme function as compared to control cells (Fig. 2, *B*). Furthermore, we studied the effect of glucose deprivation on the expression of *CD70/TNFSF7* gene, which encodes a ligand molecule for TNFRSF27/CD27, which can specifically activate TRAIL receptors and induce proliferation. As shown in Fig. 2, *C*, *TNFSF7* gene expression is resistant to glucose deprivation in control glioma cells, but inhibition of IRE1 introduces sensitivity of this gene expression to this experimental condition and strongly up-regulates TNFSF7 mRNA level.

We next tested whether glucose deprivation can affect the expression level of TNFα-induced proteins. We demonstrate that glucose deprivation does not change significantly both TNFAIP1 and TNFAIP3 mRNA levels in control glioma cells, but inhibition of IRE1 changes the expression of both these genes, but in opposite ways (Fig. 3, *A* and *B*). Thus, treatment of glioma cells harboring dn-IRE1 by glucose deprivation decreases the expression level of TNFAIP1 mRNA (-27%) and slightly increases TNFAIP3 mRNA (+15%). It is interesting to note that expression level of these mRNA is affected by IRE1 inhibition in regular growing condition (with glucose) also in opposite ways: +42% for TNFAIP1 mRNA and slightly stronger for TNFAIP3 mRNA (3.2-fold down-regulation) (Fig. 3, *A* and *B*).

As shown in Fig. 4, the inhibition of IRE1 significantly enhances the effect of glucose deprivation on the expression of *TNFRSF10B/DR5*, *TNFRSF11B*, *TNFRSF1A*, and *LITAF/PIG7* genes. We normalized here the expression levels of studied genes in control and IRE1 knockdown cells to corresponding controls and compared these relative changes.

Thereafter, we tested how IRE1 inhibition modulates the effect of glucose deprivation on the expression of LITAF/PIG7, which can mediate the TNFα expression and implicated in the TP53-induced apoptotic pathway. As shown in Fig. 3, *C*, the expression of *LITAF* gene is down-regulated upon glucose deprivation in control glioma cells, but in cells without IRE1 signaling enzyme function the expression of *LITAF* gene is significantly up-regulated (+52%) (Fig. 3, *C*). However, the inhibition of IRE1 strongly increases *LITAF* gene expression upon regular growing condition (+66%).

This study has demonstrated that the inhibition of both endoribonuclease and kinase activities of IRE1 signaling enzyme in U87 glioma cells, causes a strong (more than 8-fold) increase in the levels of TNFRSF21 mRNA, which is known as death receptor 6, and introduces sensitivity of these cells to glucose deprivation (Fig. 1, *A*). Thus, this gene resistance to glucose deprivation in control glioma cells is possibly mediated by IRE1 (Fig. 1, *A*). Moreover, the changes observed in the above studied gene, which has relation to TNF-directed apoptosis, correlate well with slower cell proliferation in cells harboring dn-IRE1 [10, 18, 48], attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth and cell survival [2, 3, 6]. There is data that TNFRSF21/DR6 induced apoptosis through a new pathway that is different from the type I and type II pathways through interacting with Bax protein [19]. Moreover, B-cells lacking TNFRSF21/DR6 show the increased proliferation rate and cell survival upon mitogenic stimulation [25]. However, this gene is highly expressed in many tumor cell lines and tumor samples [25, 26]. Thus, the role of TNFRSF21/DR6 as an apoptosis-inducing receptor is less clear and perhaps cell type dependent and needs further investigation.

Another death receptor, DR5/TNFRSF10B, is a receptor for TNF-related apoptosis-inducing ligand

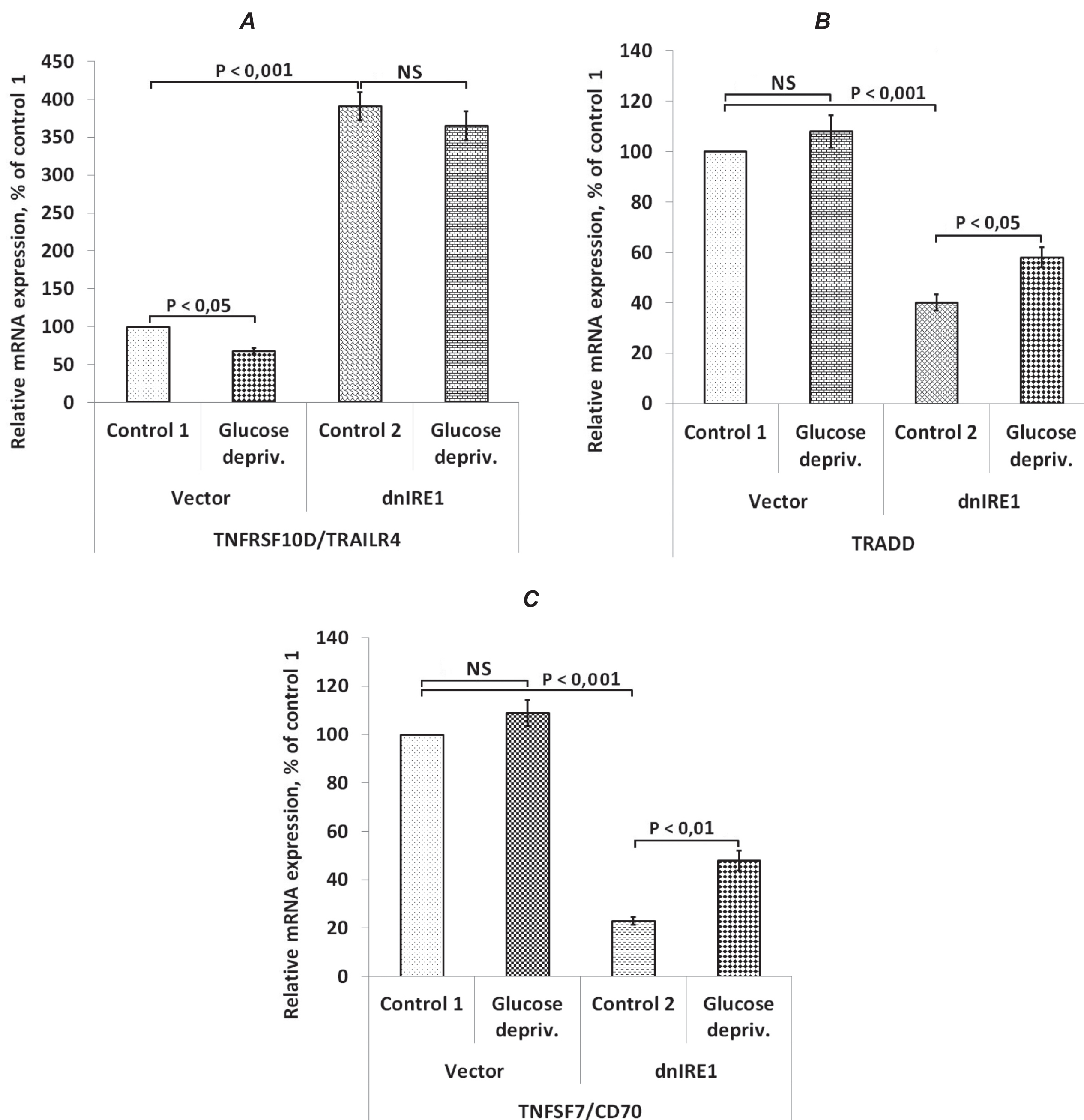

Fig. 2. Effect of glucose deprivation condition on the expression levels of TNFRSF10D (A), TRADD (B), and TNFSF7/CD70 (C) mRNAs (by qPCR) in glioma cells with a deficiency of IRE1 (dn-IRE1). These mRNA expressions values were normalized to β-actin mRNA expression and represented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4

(TRAILR2) and can initiate the extrinsic apoptotic pathway characterized by the recruitment of death domains, assembly of the death-inducing signaling complex (DISC), caspase activation and ultimately apoptosis. At the same time, in this study we have shown that TNFRSF10B/DR5 mRNA expression is up-regulated upon glucose deprivation in both types of glioma cells and down-regulated by inhibition of IRE1 signaling enzyme (Fig. 1, *B*). Thus, the changes observed in the TNFRSF10B gene as the apoptosis initiator do not correlate well with slower cell proliferation in cells harboring dn-IRE1. It is possible that TNFRSF10B/DR5 facilitates only stress-mediated apoptosis of endoplasmic reticulum, and inhibi-

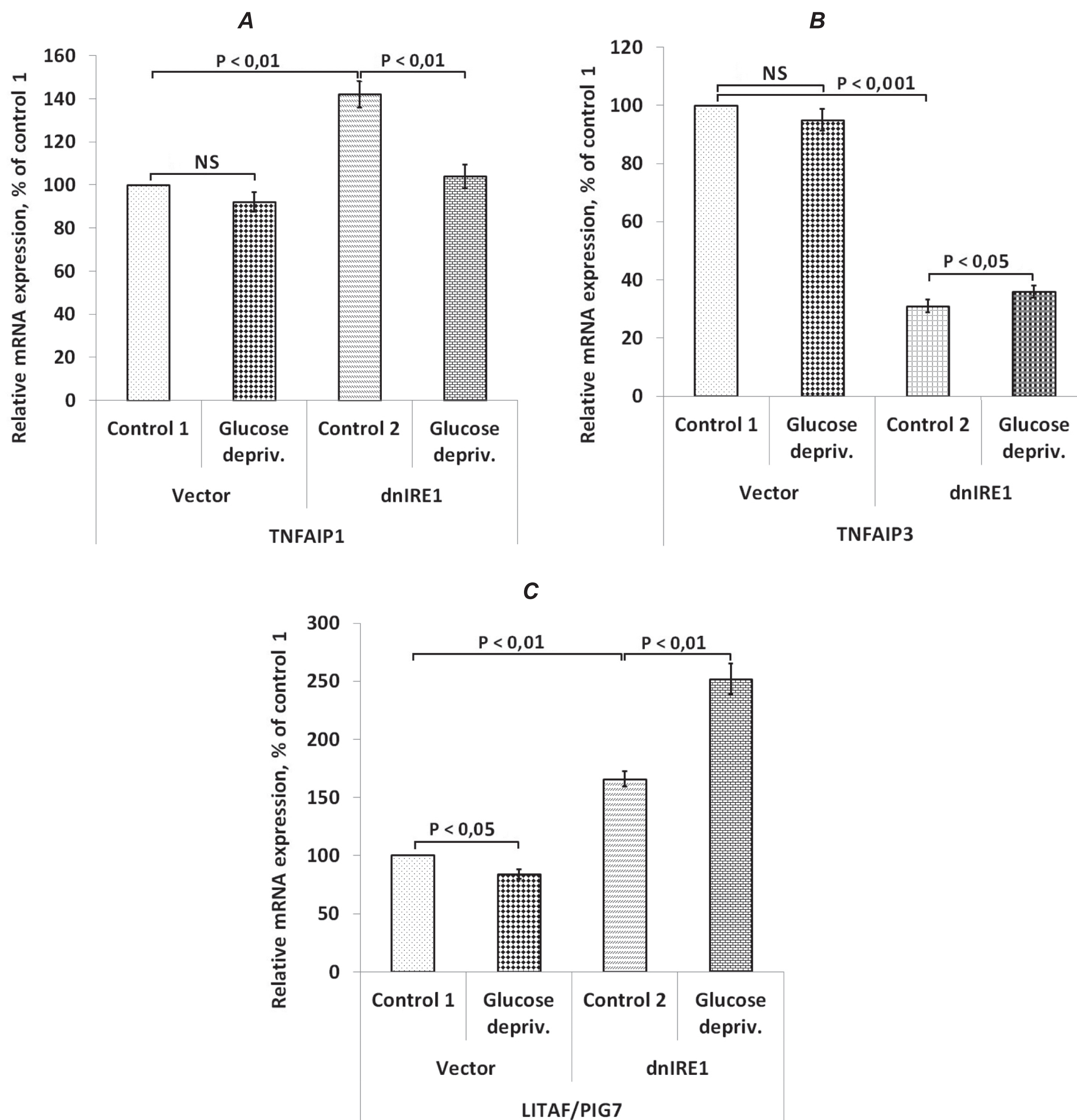

Fig. 3. Effect of glucose deprivation condition on the expression levels of TNFAIP1 (A), TNFAIP3 (B), and PIG7/LITAF (C) mRNAs (by qPCR) in glioma cells with a deficiency of IRE1 (dn-IRE1). These mRNA expressions values were normalized to β-actin mRNA expression and represented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4

tion IRE1 signaling causes down-regulation of this gene expression, because it was recently shown that DDIT3 and KAT2A proteins regulate TNFRSF10B expression in endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells [20]. However, glucose deprivation has an opposite effect on this gene expression. Recently it was also shown that TNFRSF10B/TRAILR2 inhibits maturation of the microRNA let-7 and increases proliferation of pancreatic cancer cell lines and other tumor cells [22]. Moreover, the expression of TRAIL receptors is greatly elevated in many cancer types, suggesting that tumors benefit from the expression of these receptors [25, 26]. To date, the majority of studies on

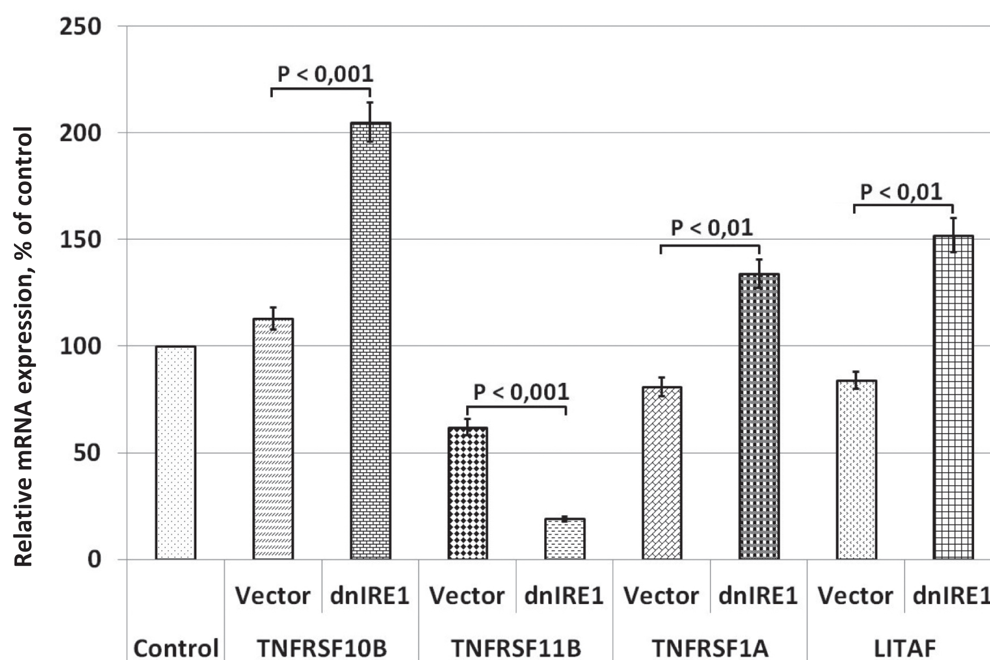

Fig. 4. IRE1 inhibition modulates the effect of glucose deprivation condition on the expression of TNFRSF10B, TNFRSF11B, TNFRSF1A, and PIG7/LITAF mRNA in glioma cells (by qPCR). These mRNA expressions values were normalized to β-actin mRNA expression and represented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4

TRAIL receptors have explored the role of these receptors as initiators of apoptosis. However, sporadic reports also suggest that TRAIL receptors can lead to other outcomes such as cytokine and chemokine production, cell proliferation and cell migration. Indeed, although transformed cells frequently express TRAIL, most do not undergo apoptosis upon engagement of these receptors and significant effort has been devoted toward exploring how to sensitize such cells to the pro-apoptotic effects of death receptor stimulation [25-29].

At the same time, we have observed a significant up-regulation of TRAILR4/TNFRSF10D in glioma cells upon inhibition of IRE1 and down-regulation upon glucose deprivation only in control glioma cells, because the inhibition of IRE1 eliminates this effect (Fig. 2, *A*). In this case we also have opposite effects of glucose deprivation and IRE1 inhibition. There is data that TNFRSF10D lacking the pro-apoptotic death domain, cannot induce apoptosis and has been shown to play an inhibitory role in TRAIL-induced cell apoptosis [21, 24]. The induction of TNFRSF10D mRNA in glioma cells upon inhibition of IRE1 correlates with down-regulation of TNFRSF10B/DR5. Similar negative correlation was observed in breast cancer cells treated by short-hairpin RNA for suppression of adenine nucleotide translocase-2 (ANT2) [49]. The suppression of ANT2 restores susceptibility of breast cancer cells to TRAIL-induced apoptosis by activating JNK and modulating TRAIL receptor expression: up-regulating the expression of TRAIL death receptors 4 and 5 (DR4 and DR5) and down-regulating the TRAILR4 [49]. Interestingly, the silencing of TNFRSF10D is related to melanoma genesis [50, 51]. It is possible that TRAILR4/TNFRSF10D as well as TRAILR2/TNFRSF10B has relation to regulation of endoplasmic reticulum stress-mediated apoptosis. Moreover, modulation of CCAAT/enhancer binding protein homologous protein (CHOP)-dependent TRAILR2/TNFRSF10B/DR5 expression by nelfinavir sensitizes glioblastoma multiform cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [52]. Therefore, a better understanding of the mechanisms underlying TRAILR4/TNFRSF10D as well as TRAILR2/TNFRSF10B is required.

Glucose deprivation also down-regulates the expression of *TNFRSF1A* and *TNFRSF11B* genes in control glioma cells; however, the inhibition of IRE1 modifies this effect: induces *TNFRSF1A* and strong-

ly reduces *TNFRSF11B* gene expressions (Fig. 1, *C* and *D*). Thus, the inhibition of IRE1 up-regulates *TNFRSF1A* and possibly contributes to suppressed cell proliferation [10, 18]. At the same time, the suppression of *TNFRSF11B* gene in glioma cells without IRE1 function is not clear yet and warrants further study.

We have also demonstrated that the expression of TRADD (TNFRSF1A-associated via death domain) is significantly up-regulated upon glucose deprivation, but only in glioma cells when IRE1 function is inhibited (Fig. 2, *B*). However, inhibition of IRE1 down-regulated this gene expression in glioma cells in regular growing condition. There is data that TRADD protein is adaptor molecule, which interacts with TNFRSF1A and participates in programmed cell death signaling. It is interesting to note that our results are consistent with recent data of Witort et al. [32] about induction of apoptotic cell death after knockdown of *TRADD* gene expression by an antisense oligonucleotide. Moreover, Shukla et al. [33] have recently shown that microRNA-30c-2-3p down-regulates TRADD and CCNE1 in breast cancer and leads to negative regulation of cell cycle progression. Thus, our data about the suppression of *TRADD* gene expression in IRE1 knockdown glioma cells may contribute to the suppression of proliferation of these cells as well as glioma growth by increased cell death and negative regulation of cell cycle progression. However, the inhibition of IRE1 modifies the sensitivity of *TRADD* gene expression to glucose deprivation.

An important role in TNFα signaling is played by a cytokine TNFSF7/CD70, a ligand for TNFRSF27/CD27, which can specifically activate of TRAIL receptors and induce proliferation [36, 37]. Its expression is significantly increased in renal cell carcinoma relative to normal kidney tissue [38]. This data has confirmed our results. We have shown that IRE1 inhibition strongly down-regulated *TNFSF7/CD70* gene expression in glioma cells and that glucose deprivation had additional suppressive effect (Fig. 2, *C*). Thus, down-regulation of this gene expression should contribute to suppression of cell proliferation and tumor growth.

It is interesting to note that the inhibition of IRE1 via dn-IRE1 overexpression up-regulates the expression of lipopolysaccharide-induced TNFα factor gene (LITAF), which encodes an important factor in the regulation of the TNFα expression by direct binding to the promoter region and is induced by TP53; however, glucose deprivation has opposite effects: down-regulates in control glioma cells and up-regulates in cells with inhibited IRE1 (Fig. 3, *C*). These results correlate well with our previous data that the inhibition of IRE1 up-regulates TP53 expression [48] and glucose deprivation can contribute to this pro-apoptotic effect of IRE1 inhibition. Thus, an increased expression of *LITAF* gene can induce TNFα expression and TNFα-mediated apoptosis. Furthermore, this data correlates well with changes in the expression profile of TNFα-induced genes (increased expression of TNFAIP1 and decreased expression of TNFAIP3 in glioma cells harboring dn-IRE1 (Fig. 3, *A* and *B*). Thus, TNFAIP1 is pro-apoptotic protein, which is decreased in cancer cells and regulates apoptosis through interaction with tumor suppressor RhoB [40]. At the same time, TNFAIP3 is a zinc finger protein and ubiquitin-editing enzyme, which preferentially inhibits TNF-mediated apoptosis and its expression increased in a number of solid human tumors [41, 42]. Thus, this data also correlates well with slower cell proliferation in cells harboring dn-IRE1 [10, 18, 48]. Endoplasmic reticulum stress also mediates both apoptosis and autophagy induced by cyclosporine A in malignant glioma cells via mTOR/p70S6K1 pathway [53].

In conclusion, glucose deprivation suppresses the expression of *TNFRSF1A*, *TNFRSF11B*, *TNFRSF10D*, and *LITAF* genes in control glioma cells and induces only *TNFRSF10B/DR5* gene, but the expression of other studied genes is resistant to this experimental condition. However, inhibition of IRE1 signaling enzyme modifies sensitivity of all studied genes to glucose deprivation. These results clearly demonstrate that the expression of TNF receptor superfamily members and TNF-inducible proteins, which are important for control of apoptosis and proliferation, is responsive to glucose deprivation preferentially through IRE1 signaling pathway of endoplasmic reticulum stress, but the mechanisms of its activation or deactivation are variable. Moreover, these gene expressions are also responsible to IRE1 inhibition upon regular growing condition and possibly contribute to suppression of tumor growth by induction of apoptosis and inhibition of cell proliferation. Thus, the changes observed in the above TNF-related factors and receptors correlate well with slower cell proliferation in cells harboring dn-IRE1, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant

tumor growth and cell survival and targeting of the unfolded protein response is a perspective way in cancer therapy [2, 3, 6, 11, 54, 55].

ІНГІБУВАННЯ IRE1 МОДИФІКУЄ ЕФЕКТ ДЕФІЦИТУ ГЛЮКОЗИ НА ЕКСПРЕСІЮ ГЕНІВ, ЩО МАЮТЬ ВІДНОШЕННЯ ДО TNFα, У КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87

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Пригнічення IRE1 (залежного від інозитолу ензиму-1), основного сигнального шляху стресу ендоплазматичного ретикулума, істотно знижує рівень проліферації клітин та ріст гліоми. Ми вивчали експресію генів, що мають відношення до TNFα, і ефект дефіциту глюкози на експресію цих генів у клітинах гліоми лінії U87, що експресують домінант-негативну IRE1, дефективну як за активністю кінази, так і ендорибонуклеази (dn-IRE1) з надією прояснити їх внесок у опосередкований IRE1 ріст гліоми. Встановлено, що за умов дефіциту глюкози спостерігається зниження експресії генів *TNFRSF11B*, *TNFRSF1A*, *TNFRSF10D/TRAILR4* та *LITAF* і посилення гена – *TNFRSF10B/TRAILR2/DR5* на рівні мРНК у контрольних клітинах гліоми. Водночас, експресія генів *TNFRSF21/DR6*, *TNFAIP1*, *TNFAIP3*, *TRADD* та *CD70/TNFSF7* у контрольних клітинах гліоми виявилася резистентною до умов дефіциту глюкози, але пригнічення IRE1 модифікувало ефект дефіциту глюкози на експресію генів *LITAF*, *TNFRSF21*, *TNFRSF11B* і *TRADD* та індукувало чутливість експресії генів *TNFRSF10B*, *TNFRSF1A* та *CD70* до умов дефіциту глюкози. Ми також показали, що експресія всіх досліджених генів у клітинах гліоми змінювалась за пригнічення IRE1, за виключенням гена *TNFRSF1A* (за порівняння з контрольними клітинами гліоми). Більше того, зміни в експресії генів *TNFRSF1A*, *TNFRSF10D/TRAILR4* та *LITAF*, що індукуються за умов дефіциту глюкози, мали протилежну спрямованість до тих, що спостерігаються за пригнічення IRE1. Результати цієї роботи продемонстрували, що рівень експресії генів протеїнів, що індукуються TNFα, та суперродини рецепторів TNF, які мають відношення до смерті клітин і їх проліферації, регулюються IRE1, ефектором стресу ендоплазматичного ретикулума, а також геноспецифічно залежать від дефіциту глюкози. Таким чином, пригнічення активності кінази та ендорибонуклеази IRE1 корелює зі зниженням росту пухлини і дерегуляцією експресії генів протеїнів, що індукуються TNFα та суперродиною рецепторів TNF специфічно до кожного із генів.

К л ю ч о в і с л о в а: експресія мРНК, стрес ендоплазматичного ретикулума, LITAF, TNFAIP1, TNFAIP3, TNFRSF21/DR6, TNFRSF10B/DR5, TNFRSF10D, TNFRSF11B, TNFRSF1A, TRADD, пригнічення IRE, клітини гліоми, дефіцит глюкози.

ИНГИБИРОВАНИЕ IRE1 МОДИФИЦИРУЕТ ЭФФЕКТ ДЕФИЦИТА ГЛЮКОЗЫ НА ЭКСПРЕССИЮ ГЕНОВ, ИМЕЮЩИХ ОТНОШЕНИЕ К TNFα, В КЛЕТКАХ ГЛИОМЫ ЛИНИИ U87

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Угнетение IRE1 (зависимого от инозитола энзима-1), основного сигнального пути стресса эндоплазматического ретикулума, существенно снижает уровень пролиферации клеток и рост глиомы. Мы изучали экспрессию генов, имеющих отношение к TNFα, и эффект дефицита глюкозы на экспрессию этих генов в клетках глиомы линии U87, которые экспрессируют доминант-негативный IRE1, дефективный как по активности киназы, так и эндорибонуклеазы (dn-IRE1) с надеждой выяснить их вклад в опосредованный IRE1 рост глиомы. Установлено, что при дефиците глюкозы наблюдается снижение экспрессии генов *TNFRSF11B*, *TNFRSF1A*, *TNFRSF10D/TRAILR4* и *LITAF* и усиление гена – *TNFRSF10B/TRAILR2/DR5* на уровне мРНК в

контрольных клетках глиомы. В то же время, экспрессия генов *TNFRSF21/DR6*, *TNFAIP1*, *TNFAIP3*, *TRADD* и *CD70/TNFSF7* в контрольных клетках глиомы была резистентной к дефициту глюкозы, но угнетение IRE1 модифицировало эффект дефицита глюкозы на экспрессию генов *LITAF*, *TNFRSF21*, *TNFRSF11B* и *TRADD* и индуцировало чувствительность экспрессии генов *TNFRSF10B*, *TNFRSF1A* и *CD70* к условиям дефицита глюкозы. Мы также показали, что экспрессия всех исследованных генов в клетках глиомы изменялась при угнетении IRE1, за исключением гена *TNFRSF1A* (по сравнению с контрольными клетками глиомы). Более того, изменения в экспрессии генов *TNFRSF1A*, *TNFRSF10D/TRAILR4* и *LITAF*, которые индуцируются в условиях дефицита глюкозы, имели противоположную направленность по отношению к тем, которые наблюдаются при угнетении IRE1. Результаты этой работы продемонстрировали, что уровень экспрессии генов протеинов, индуцируемых TNFα, и суперсемейства рецепторов TNF, которые имеют отношение к смерти клеток и их пролиферации, регулируется IRE1, эффектором стресса эндоплазматического ретикулума, а также геноспецифически зависит от дефицита глюкозы. Таким образом, угнетение активности киназы и эндорибонуклеазы IRE1 коррелирует со снижением роста опухоли и с дисрегуляцией экспрессии генов протеинов, индуцируемых TNFα и суперсемейством рецепторов TNF специфично к каждому из генов.

Ключевые слова: экспрессия мРНК, стресс эндоплазматического ретикулума, LITAF, TNFAIP1, TNFAIP3, TNFRSF21/DR6, TNFRSF10B/DR5, TNFRSF10D, TNFRSF11B, TNFRSF1A, TRADD, угнетение IRE, клетки глиомы, дефицит глюкозы.

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