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Hypoxic regulation of *PFKFB*-3 and *PFKFB*-4 gene expression in gastric and pancreatic cancer cell lines and expression of *PFKFB* genes in gastric cancers

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Previously we have shown that hypoxia strongly induces the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 (*PFKFB*-3 and *PFKFB*-4) genes in several cancer cell lines *via* a HIF-dependent mechanism. In this paper we studied the expression and hypoxic regulation of *PFKFB*-4 and *PFKFB*-3 mRNA as well as its correlation with *HIF-1α*, *HIF-2α*, *VEGF* and *Glut1* mRNA expression in the pancreatic cancer cell line Panc1 and two gastric cancer cell lines MKN45 and NUGC3. This study clearly demonstrated that *PFKFB*-3 and *PFKFB*-4 mRNA are expresses in MKN45, NUGC3 and Panc1 cancers cells and that both genes are responsive to hypoxia *in vitro*. However, their basal level of expression and hypoxia responsiveness vary in the different cells studied. Particularly, *PFKFB*-3 mRNA is highly expressed in MKN45 and NUGC3 cancer cells, with the highest response to hypoxia in the NUGC3 cell line. The *PFKFB*-4 mRNA has a variable low basal level of expression in both gastric and pancreatic cancer cell lines. However, the highest hypoxia response of *PFKFB*-4 mRNA is found in the pancreatic cancer cell line Panc1. The basal level of PFKFB-4 protein expression is the highest in NUGC3 gastric cancer cell line and lowest in Panc1 cells, with the highest response to hypoxia in the pancreatic cancer cell line. Further studies showed that *PFKFB*-3 and *PFKFB*-4 gene expression was highly responsive to the hypoxia mimic dimethyloxalylglycine, a specific inhibitor of HIF-α hydroxylase enzymes, suggesting that the hypoxia responsiveness of *PFKFB*-3 and *PFKFB*-4 genes in these cell lines is regulated by the HIF transcription complex. The expression of *VEGF* and *Glut*1, which are known HIF-dependent genes, is also strongly induced under hypoxic conditions in gastric and pancreatic cancer cell lines. The levels of HIF-1α protein are increased in both gastric and pancreatic cancer cell lines under hypoxic conditions. However, the basal level of HIF-1α as well as HIF-2α mRNA expression and their hypoxia responsiveness are different in the MKN45 and NUGC3 cancer cells. Thus, the expression of HIF-1α mRNA is decreased in both gastric cancer cell lines treated by hypoxia or dimethyloxalylglycine, but HIF-2α mRNA expression is not changed significantly in NUGC3 and slightly increased in MKN45 cells. Expression of *PFKFB*-4 and *PFKFB*-3 was also studied in gastric cancers and corresponding nonmalignant tissue counterparts from the same patients on both the mRNA and protein levels. The expression of *PFKFB*-3 and *PFKFB*-4 mRNA as well as *PFKFB*-1 and *PFKFB*-2 mRNA was observed in normal human gastric tissue and was increased in malignant gastric tumors. The basal level of PFKFB-4 protein expression in gastric cancers was much higher as compared to the PFKFB-3 isoenzyme. In conclusion, this study provides evidence that *PFKFB*-4 and *PFKFB*-3 genes are also expressed in gastric and pan-

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Abbreviations: Glut1, glucose transporter 1; HIF, hypoxia-inducible factor; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; VEGF, vascular endothelial growth factor.

creatic cancer cells, they strongly respond to hypoxia *via* a HIF-1α dependent mechanism and, together with the expression of *PFKFB*-1 and *PFKFB*-2 genes, possibly have a significant role in the Warburg effect which is found in malignant cells.

Keywords: PFKFB-3, PFKFB-4, hypoxia, HIF, Panc1, MKN-45, gastric cancer

INTRODUCTION

Hypoxia sensing and related signalling events, including activation of hypoxia-inducible factor 1 (HIF-1), represent key features in cell biochemistry, physiology and molecular biology because it is an important component of many physiological and pathophysiological processes, including tumor formation and growth (Semenza, 2000; 2002; Hockel & Vaupel, 2001; Wykoff *et al.*, 2001; Wenger, 2002; Lu *et al.*, 2002; Bruick, 2003). Most tumors are usually exposed to a hypoxic microenvironment due to their irregular growth and insufficient blood supply while pancreatic tumors have enhanced vascular supply (Vaupel 1996; Dang & Semenza, 1999; Hockel & Vaupel, 2001). Important in adaptations to hypoxia is the activation of genes that ameliorate or compensate for the oxygen deficit, especially of genes involved in glycolysis and genes that facilitate glucose transport (Brown & Giaccia, 1998; Gleade & Ratcliffe, 1998; Dang & Semenza, 1999; Seagroves *et al.*, 2000; Lu *et al.*, 2002; Wenger, 2002).

The transcription factor HIF is central in coordinating many of the transcriptional adaptations to hypoxia and a necessary mediator of the hypoxic effect in mammalian cells (Wenger, 2002; Bitlon & Booker, 2003; Greijer *et al.*, 2005). HIF is a heterodimeric transcription factor composed of two subunits: a constitutively expressed β-subunit and an α-subunit. Their expression and activity are controlled by intracellular oxygen concentration (Wenger, 2002). In mammals HIF-α subunit exists as multiple isoforms with different biological properties. Three principal isoforms (HIF-1α, HIF-2α and HIF-3α) are encoded by three distinct genetic loci, further diversity being generated by alternative promoter usage and alternative splicing (Semenza *et al.*, 2001; Makino *et al.*, 2001; 2002). Expression of these genes varies in different organs and cell types but *HIF-1α* is the widely expressed and major functional isoform (Bitlon & Booker, 2003; Hu *et al.*, 2003; Huang & Bunn, 2003; Sowter *et al.*, 2003; Elvidge *et al.*, 2006). The HIF-1α and HIF-2α isoforms are closely related, but HIF-3α is significantly different and is expressed as a number of alternative spliced variants (Makino *et al.*, 2001). One of them, inhibitory PAS domain protein (IPAS), is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3α locus. It is a negative regulator of hypoxia-inducible gene expression. IPAS functions as a dominant negative regulator of hypoxia-inducible transcription factors by forming complexes with those proteins that fail to bind to hypoxia response elements of target genes. The alternatively spliced transcript of HIF-3α was only observed in the heart and lungs under hypoxic conditions (6% oxygen for 6 h), thus defining a novel mechanism of hypoxia-dependent regulation of gene expression (Makino *et al.*, 2002). Importantly, this mechanism may establish a negative feedback loop regulation of adaptive responses to hypoxia/ischemia in these tissues. There are also several alternative spliced variants of HIF-1α (HIF-1α^{516}, HIF-1α^{557}, HIF-1α^{735}) that terminate translation respectively at codons 516, 557 and 735, resulting in the absence of both N-activation domain and C-activation domain or C-activation domain only (Wenger, 2002). There are data that overexpression of an exogenous testis-specific splice isoform of human HIF-1α (hHIF-1αTe) resulted in the inhibition of the endogenous HIF-1 transcriptional activity, demonstrating that the testis-specific hHIF-1αTe isoform is a dominant-negative regulator of normal HIF-1 activity (Depping *et al.*, 2004).

The expression of the transcriptional complex HIF is tightly coupled to oxygen concentration. Whereas the HIF-1β subunit is constitutively expressed, HIF-1α is highly unstable in normoxic conditions, being rapidly degraded by the ubiquitin-proteasome system (Epstein *et al.*, 2001; Ivan *et al.*, 2001; Metzen & Ratcliffe, 2004). Recently, the oxygen sensors monitoring this hypoxic response were identified as prolyl- and asparaginyl-hydroxylase enzymes, which during normoxia (normal physiological levels of oxygen) mediate the rapid degradation of HIF-α and prevent transcriptional recruitment of the cofactor CBP/p300, respectively (Epstein *et al.*, 2001; Ivan *et al.*, 2001; Metzen & Ratcliffe, 2004). HIF-1 activation depends on the hydroxylation of specific prolyl and asparaginyl residues in the α subunit of HIF complex that controls the survival and transcriptional activity of this protein (Ivan *et al.*, 2001; Min *et al.*, 2002; Schofield & Ratcliffe, 2004). These recently described HIF hydroxylases are a family of non-haem iron and oxoglutarate-dependent dioxygenases that define a novel mechanism of protein modification that transduces the oxygen-sensing signal and controls hypoxic gene activation (Semenza, 2001; Lando *et al.*, 2002; Min *et al.*, 2002; Masson & Ratcliffe, 2003; Mole *et al.*, 2003; Appelhoff *et al.*, 2004). There is data (Goyal *et al.*, 2004) that upregulation of NADPH oxidase 1 under hypoxia activates hypoxia-inducible factor 1 *via* an increase in reactive oxygen species. The HIF pathway is also responsible for patterns of gene expression as well as cell growth and angiogenesis in cancer (Wykoff *et al.*, 2001; Lu *et al.*, 2002; Hopfl *et al.*, 2004; Stoeltzing *et al.*, 2004; Minchenko *et al.*, 2005b).

The transcriptional complex HIF binds to a specific hypoxia-responsive element in the regulatory regions of genes whose expression is regulated by hypoxia (Ratcliffe *et al.*, 1998; Minchenko & Caro, 2000; Wenger, 2002; Bitlon & Booker, 2003; Greijer *et al.*, 2005; Manalo *et al.*, 2005). The hypoxia-responsive element has been identified now in various hypoxia-responsive genes (Gleade & Ratcliffe, 1998; Wenger, 2002). The hypoxia-responsible element/enhancer mediates hypoxic induction by recruiting the HIF complex and allowing its interaction with other trans-activators and the basal transcriptional machinery (Epstein *et al.*, 2001). Recently, a hypoxia-responsive element was identified in the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase genes *PFKFB*-4 and *PFKFB*-3 (Minchenko *et al.*, 2004; Fukasawa *et al.*, 2004; Obach *et al.*, 2004).

Fructose-2,6-bisphosphate is considered to be the major allosteric activator of 6-phosphofructo-1-kinase, which is a rate-limiting enzyme of glycolysis, and an inhibitor of fructose-1,6-bisphosphatase (Okar *et al.*, 2001; Hue *et al.*, 2003). The PFKFB (EC 2.7.1.105/EC 3.1.3.46) enzyme is a metabolic signaling polypeptide which is responsible for maintaining the cellular levels of fructose-2,6-bisphosphate (Pilkis *et al.*, 1995; Okar *et al.*, 2001). A family of bifunctional PFKFB enzymes controls fructose-2,6-bisphosphate level and glycolysis (Pilkis *et al.*, 1995; Okar *et al.*, 2001). Four different genes encode isoenzymes of PFKFB that differ in their kinetic and regulatory properties (Pilkis *et al.*, 1995; Sakakibara *et al.*, 1999; Okar *et al.*, 2001; Rider *et al.*, 2004). The mammalian *PFKFB*-4 gene encodes an isoenzyme which originally was identified in the testes (Sakata *et al.*, 1991; Manzano *et al.*, 1998). Importantly, most cells express more than one isoform (Sakakibara *et al.*, 1999; Minchenko *et al.*, 2003; 2004; 2005c).

Thus, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is a key regulatory enzyme of glycolysis both in normal and hypoxic conditions because hypoxia induces the expression of *PFKFB*-1, *PFKFB*-2, *PFKFB*-3 and *PFKFB*-4 genes in various cell lines (Chesney *et al.*, 1999; Marsin *et al.*, 2002; Minchenko *et al.*, 2002; 2003; 2004; 2005c). However, regulation of the expression of these PFKFB isoenzymes following hypoxic treatment is different and occurrs in a cell-specific manner. Moreover, the rapid activation of glycolysis by fructose-2,6-bisphosphate as well as by hypoxia in activated monocytes is regulated by phosphorylation - dephosphorylation of PFKFB isoenzymes at the N- or C-terminus by AMP-activated and several other protein kinases in different signaling pathways (Marsin *et al.*, 2002; Hue *et al.*, 2003; Rider *et al.*, 2004). High expression of PFKFB-4, PFKFB-3 and its inducible isoform was observed in various human cancers (Chesney *et al.*, 1999; Atsumi *et al.*, 2002; Minchenko *et al.*, 2004; 2005a; 2005b). Moreover, there is data supporting a potential role for the phosphorylation of PFKFB-3 protein in enhanced glycolysis, the progression of cancer and angiogenesis (Bando *et al.*, 2005). Highly phosphorylated PFKFB-3 was found in human tumor cells, vascular endothelial cells, and smooth muscle cells, as determined by immunostaining with an anti-phospho-PFK-2 (PFKFB-3) antibody (Bando *et al.*, 2005). However, little is known about the expression and hypoxia-responsiveness of *PFKFB*-4 and *PFKFB*-3 in pancreatic and gastric cancer cells which significantly differ from many other malignant cell lines.

This study provides evidence that the *PFKFB*-4 and *PFKFB*-3 genes are expressed in human pancreatic and gastric cancer cell lines, strongly respond to hypoxia *via* an HIF-dependent mechanism and are overexpressed in gastric malignant tumors. Hypoxic induction of HIF-1α protein in these cell lines correlates with reduction *HIF-1α* mRNA expression.

MATERIALS AND METHODS

Materials. Dimethyloxalylglycine was obtained from Frontier Scientific, Inc. (Logan, UT, USA). [α^{32}P]UTP (800 Ci/mmol) and Hyperfilm ECL were obtained from Amersham Biosciences.

Cell lines and culture conditions. Human gastric cancer cell lines MKN45 and NUGC3 as well as pancreatic cancer cell line Pank1 were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to the supplier's protocols. The cells were incubated at 37°C before harvesting under normoxic (21% oxygen and 5% carbon dioxide) or hypoxic (1% oxygen, 5% carbon dioxide and 94% nitrogen for 6 h) conditions or exposed for 6 h to 1 mM dimethyloxalylglycine.

RNA isolation. Total RNA was extracted using Trizol reagent according to the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). RNA pellet was washed with 75% ethanol, dissolved in nuclease-free water and used for ribonuclease protection assays.

Plasmid construction. The plasmids used for the determination of *PFKFB*-1, *PFKFB*-2, *PFKFB*-3, *PFKFB*-4, HIF-1α, VEGF, and Glut1 mRNA and 18S ribosomal RNA have been described (Minchenko *et al.*, 2002; 2003; 2005a). The human HIF-2α cDNA was synthesized by RT-PCR using total RNA from human breast adenocarcinoma cell line SKBR-3 and oligo(dT). For first-strand cDNA synthesis Sensiscript RT Kit (QIAGEN, Germany) was used. PCR amplification was performed with the following oligonucleotides: 5′-GTGGCGTCTCCCTCGCAGAG-3′

(forward primer) and 3'-CTCCAAGCTCACGAC-CTTGG-5' (reverse primer) using HotStarTaq Master Mix Kit (QIAGEN). These oligonucleotides correspond to nucleotide sequences 3387–3406 and 4205–4224 of human HIF-2α cDNA (GenBank accession number BC051338). The HIF-2α cDNA was cloned into pCRII-TOPO cloning vector (Invitrogen, USA). The shorter (410 bp) *Eco*RI–*Bgl*II fragment of HIF-2α cDNA was recloned into *Eco*RI and *Bam*HI sites of pBluescript II SK$^+$ (Stratagene, USA) and used for syntheses of antisense probe for ribonuclease protection assays of HIF-2α mRNA. The probe was verified by sequencing the insert in the plasmid. The 18S rRNA antisense probe was used to ensure equal loading of total RNA.

***In vitro* transcription to prepare antisense probes for ribonuclease protection assay**. The synthesis of radiolabeled probes for ribonuclease protection assay was according to the BD Biosciences protocol using T7 RNA polymerase (BD Biosciences Pharmingen, San Diego, CA, USA) and [α^{32}P]UTP. For ribonuclease protection assays water solutions of total RNA were dried under vacuum and dissolved in 20 µl of 80% formamide hybridization buffer containing labeled probes. Samples were preincubated for 5 min at 85°C to denature RNA and then incubated for 16 h at 45°C as described previously (Minchenko & Caro, 2000). Single-strand RNA was removed by digestion with ribonuclease T1 at 30°C for 60 min and extracted with phenol/chloroform. Protected probe fragments were precipitated with 2.5 volumes of ethanol with the addition of 10 µg of transfer RNA/sample. The samples were denatured and protected fragments separated on a 6% polyacrylamide sequencing gel in Tris/borate/EDTA buffer for 2 h at 50 mA as described previously (Minchenko *et al.*, 1994). The expression of different mRNAs was determined using Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co., Japan). The intensity of each mRNA band was normalized for 18 S ribosomal RNA levels and results are expressed as the ratio of mRNA to 18S rRNA (percent of control).

Western blot analysis of PFKFB-4 and PFKFB-3. For the detection of PFKFB-4 and PFKFB-3 protein levels Western blot analysis was used. Cell extracts were prepared as described previously (Minchenko *et al.*, 2000). The proteins were resolved using sodium dodecyl sulfate/polyacrylamide gel (10% acrylamide) electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P Transfer Membrane; Millipore, USA) by a semi-dry blotting system. For detection of PFKFB-4 the membrane was incubated with a rabbit polyclonal anti-PFKFB-4 (1:10000 dilution) antibody as described previously (Minchenko *et al.*, 2005c). For detection of PFKFB-3 we used a goat polyclonal anti-PFKFB-3 [PFK-2br/pl (N-11); sc-100890; a dilution of 1:1000] antibody from Santa Cruz Biotechnology (USA). For Western blotting of iPFKFB-3 was used a rabbit polyclonal anti-iPFK-2 (1:1000 dilution) antibody (Atsumi *et al.*, 2002). HIF-1α expression was measured with polyclonal anti-HIF-1α antibody (mAb) (Novus Biologicals, USA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse IgG (Santa Cruz Biotechnology, USA) was used as a secondary antibody with a dilution of 1:2000.

The protein complexes were visualized by enhanced chemiluminescence reagents (Amersham Biosciences) as described previously (Minchenko *et al.*, 2004). β-Actin was used to ensure equal loading of the samples.

Statistical analysis. The results are expressed as mean ± standard error of the mean (S.E.M.) of four independent experiments. Comparison of two means was performed by the use of unpaired Student's *t*-test. *P* value of <0.05 was regarded as significant.

RESULTS

In this work, we studies the expression, hypoxic regulation and mechanisms of hypoxic regulation of *PFKFB*-3, *PFKFB*-4, *HIF*-1α, *HIF*-2α, *VEGF* and *Glut1* genes in human pancreatic and gastric cancer cell lines as well as in human gastric cancers.

Effect of hypoxia and dimethyloxalylglycine on *PFKFB*-4 and *PFKFB*-3 gene expression in gastric and pancreatic cancer cell lines

To examine the effects of hypoxia and dimethyloxalylglycine on the expression of the *PFKFB*-4 gene in gastric and pancreatic cancer cell lines, mRNA levels were measured by ribonuclease protection assays. As shown in Fig. 1, the transcript

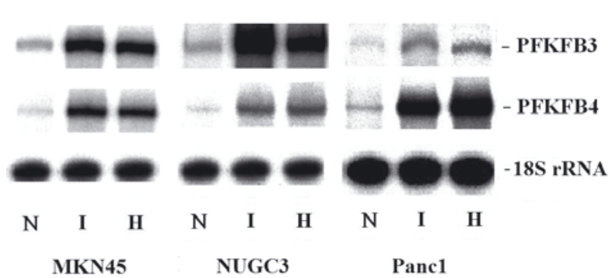

Figure 1. Effect of hypoxia and hypoxia mimic dimethyloxalylglycine on the expression of *PFKFB*-3 and *PFKFB*-4 mRNA in human gastric cancer cell lines MKN45 and NUGC3 and pancreatic cancer cell line Panc1, measured by ribonuclease protection assay.
The cells were exposed to hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h. N, control (normoxic) cells. Intensities of different mRNA bands were normalized to 18S rRNA. The data are representative of four experiments.

level of PFKFB-4 isozyme is very low but detectable in the human gastric cancer cell line MKN45 growing under normoxic conditions. These cells also express *PFKFB*-3. However, the basal transcript level of *PFKFB*-3 is much higher as compared to *PFKFB*-4. Exposure of MKN45 gastric cancer cells to hypoxia (1% oxygen for 6 h) greatly stimulated the expression of both *PFKFB*-4 and *PFKFB*-3 mRNA. The ribonuclease protection assays analysis at *PFKFB*-4 and *PFKFB*-3 mRNA expression in the MKN45 gastric cancer cells was also highly responsive to dimethyloxalylglycine.

In the human gastric cancer cell line NUGC3 the basal transcript level of *PFKFB*-4 and *PFKFB*-3 and their hypoxia responsiveness were different. Thus, the transcript level of *PFKFB*-4 mRNA in these cancer cells growing under normoxic conditions was much lower as compared to the PFKFB-3 isoform. The expression of both *PFKFB*-4 and *PFKFB*-3 mRNA in NUGC3 gastric cancer cells was increased by hypoxia; however, hypoxia responsiveness of *PFKFB*-3 was much higher as compared to *PFKFB*-4. Expression of both *PFKFB*-4 and *PFKFB*-3 mRNA in NUGC3 gastric cancer cells was highly responsive to the hypoxia mimic dimethyloxalylglycine.

We also studied the expression and hypoxia responsiveness of *PFKFB*-4 and *PFKFB*-3 genes on the mRNA level in the human pancreatic cancer cell line Panc1 (Fig. 2). The transcript level of the PFKFB-4 isoenzyme in this cancer cell line growing under normoxic conditions was low but slightly higher as compared to *PFKFB*-3 mRNA. Exposure of these cancer cells to hypoxia stimulated the expression of *PFKFB*-4 and, to a lesser extent, *PFKFB*-3 mRNA. Dimethyloxalylglycine also strongly induce the *PFKFB*-4 mRNA expression in Panc1 cells. The expression of *PFKFB*-3 gene in the pancreatic cancer cells was increased by dimethyloxalylglycine but the hypoxia responsibility of this isoform of PFKFB was much lower as compared to *PFKFB*-4 mRNA.

The results of four independent ribonuclease protection experiments were quantified using a Fujix BAS 2000 Bio-Image Analyzer and expressed as the ratio between *PFKFB*-4 or *PFKFB*-3 mRNA and 18S rRNA. As shown in Fig. 2, the increase of the *PFKFB*-4 and *PFKFB*-3 transcript levels in the MKN45 gastric cancer cells by hypoxia was similar: 406% ($P < 0.01$) and 398% ($P < 0.01$), respectively. However, in the gastric cancer cell line NUGC3 hypoxia increase of the *PFKFB*-3 transcript level more strongly that than of as compared to *PFKFB*-4: *PFKFB*-3 by 1198% ($P < 0.001$) and *PFKFB*-4 by 306% ($P < 0.01$). Quantification of ribonuclease protection assays of *PFKFB*-4 and *PFKFB*-3 mRNA levels in the pancreatic cancer cells shows that hypoxia induced *PFKFB*-4 transcript by 12-fold ($P < 0.001$) and *PFKFB*-3 mRNA level – twice ($P < 0.05$).

Further investigation showed, that hypoxia or dimethyloxalylglycine induced the expression of PFKFB4 protein measured by Western blot analysis with a rabbit polyclonal anti-PFKFB4 antibody in both gastric and pancreatic cancer cells (Fig. 3). However, both the basal and hypoxia-induced expression of PFKFB-4 protein differed in both gastric and pancreatic cancer cell lines. A strongest induction of PFKFB-4 protein level both by hypoxia and dimethyloxalylglycine was observed in the pancreatic cancer cells.

Effect of hypoxia and dimethyloxalylglycine on *HIF-1α* and *HIF-2α* gene expression in gastric and pancreatic cancer cell lines

As shown in Fig. 4, hypoxia induces the protein level of HIF-1α in the both gastric cancer cell lines MKN45 and NUGC3 as well as in the pancreatic cancer cell line Panc1. However, the basal HIF-1α protein level differs in the MKN45 and NUGC3 gastric cancer cell lines: it is much higher in the MKN45 cells. In the NUGC3 and Panc1 cell lines the basal and hypoxia inducible levels of HIF-1α protein were similar. However, the transcript levels of HIF-

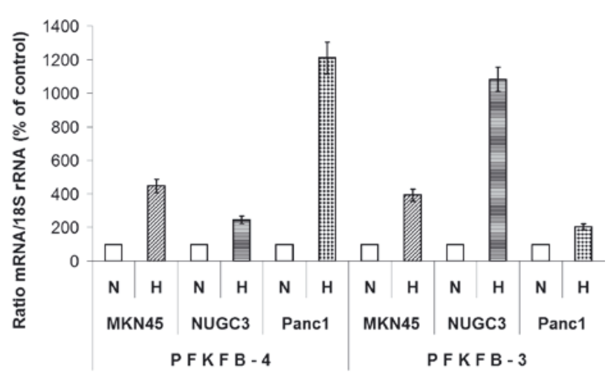

Figure 2. The effect of hypoxia on *PFKFB*-4 and *PFKFB*-3 mRNA expression, measured by ribonuclease protection assay, in human gastric cancer cell lines MKN45 and NUGC3 and pancreatic cancer cell line Panc1.
The cells were exposed to hypoxia (H) for 6 h. N, control (normoxic) cells. Intensities of different mRNA bands were normalized to 18S rRNA. The bar heights are mean values ± standard errors of the mean.

Figure 3. Western analysis of the expression of PFKFB-4 protein in human gastric cancer cell lines MKN45 and NUGC3 and in human pancreatic cancer cell line Panc1: effect of hypoxia (H) and dimethyloxalylglycine (I).
The cells were exposed to hypoxia or treated with dimethyloxalylglycine for 6 h. N, control (normoxic) cells. Intensities of PFKFB-4 protein bands were normalized to β-actin. The data are representative of four experiments.

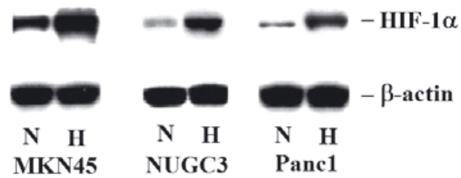

Figure 4. Effect of hypoxia on the expression of HIF-1α protein in human gastric cancer cell lines MKN45 and NUGC3 and in pancreatic cancer cell line Panc1, measured by Western analysis.
The cells were exposed to hypoxia (H) for 6 h. N, control (normoxic) cells. Intensities of HIF-1α protein bands were normalized to β-actin. The data are representative of three experiments.

1α were decreased in both gastric cancer cell lines under hypoxic conditions (Fig. 5). The MKN45 and NUGC3 gastric cancer cells treated with dimethyloxalylglycine have also decreased levels of *HIF*-1α mRNA. Analysis of HIF-2α mRNA expression and its hypoxia responsiveness in the MKN45 and NUGC3 cancer cells showed that the basal level of *HIF*-2α mRNA was much lower as compared to *HIF*-1α mRNA (Fig. 5), but showed a positive response to hypoxia or dimethyloxalylglycine treatment in MKN45, while in the NUGC3 cancer cells was not responsive to hypoxia and slightly decreased *HIF*-2α mRNA expression after dimethyloxalylglycine treatment.

Effect of hypoxia and dimethyloxalylglycine on *VEGF* and *Glut1* gene expressions in gastric and pancreatic cancer cells

We also studied the hypoxic regulation of *Glut1* and *VEGF* mRNA expression to compare the hypoxia responsiveness of the *PFKFB*-4 and *PFKFB*-3 genes with known HIF-1 dependent genes. Results of this study clearly demonstrated that hypoxia and dimethyloxalylglycine strongly induced the transcript levels of *Glut1* and *VEGF* genes both in the gastric and pancreatic cancer cell lines, like it was obtained for *PFKFB*-4 and *PFKFB*-3 genes (Fig. 6). However, some differences were found in the induction of *Glut1* and *VEGF* genes by hypoxia or dimethyloxalylglycine treatment in the MKN45 and NUGC3 gastric cancer cell lines: the expression of *Glut1* mRNA was increased much more strongly in the MKN45 cells, while the expression of *VEGF* mRNA – in the NUGC3 gastric cancer cells.

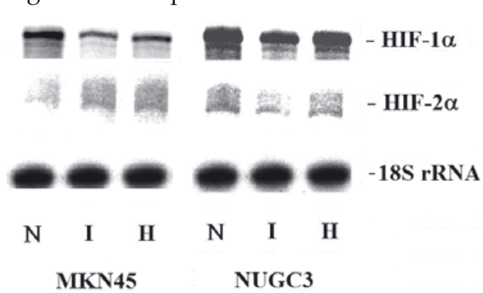

Figure 5. Effect of hypoxia and hypoxia mimic dimethyloxalylglycine on the expression of *HIF*-1α and *HIF*-2α mRNA, measured by ribonuclease protection assay, in human gastric cancer cell line MKN45 and NUGC3.
The cells were exposed to hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h. N, control (normoxic) cells. Intensities of different mRNA bands were normalized to 18S rRNA. The data are representative of three experiments.

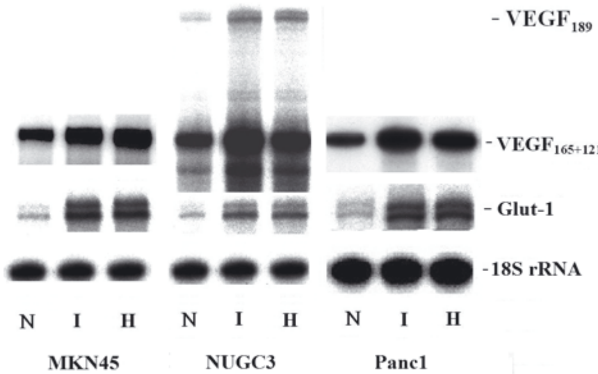

Figure 6. Effect of hypoxia and hypoxia mimic dimethyloxalylglycine on the expression of *VEGF* and *Glut1* mRNA in human gastric cancer cell lines MKN45 and NUGC3 and pancreatic cancer cell line Panc1, measured by ribonuclease protection assay.
The cells were exposed to hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h. N, control (normoxic) cells. Intensities of different mRNA bands were normalized to 18S rRNA. The data are representative of three experiments.

Expression of *PFKFB*-1, *PFKFB*-2, *PFKFB*-3, *PFKFB*-4, *Glut1* and *VEGF* mRNA in human gastric malignant tumors

The *PFKFB*-4 and *PFKFB*-3 gene expression was studied in gastric cancers and corresponding nonmalignant tissue counterparts from the same patients on both the mRNA and protein levels. Moreover, we also analyzed the expression of two other *PFKFB* genes – *PFKFB*-1 and *PFKFB*-2. As shown in

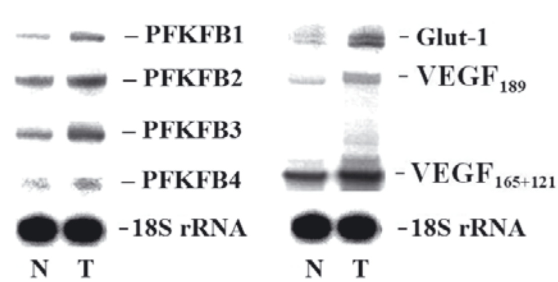

Figure 7. Expression of *PFKFB*-1, *PFKFB*-2, *PFKFB*-3, *PFKFB*-4, *Glut1* and *VEGF* mRNA in human gastric malignant tumors (T) and corresponding nonmalignant tissue (N) from the same patients, as measured by ribonuclease protection assay.
Intensities of different mRNA bands were normalized to 18S rRNA. The data are representative of three experiments.

Figure 8. Western blot analysis of PFKFB-4, PFKFB-3 and inducible PFKFB-3 (iPFKFB-3) protein expression in human gastric malignant tumors (T) and corresponding nonmalignant tissue (N) from the same patients.
Intensities PFKFB-4, PFKFB-3 and iPFKFB-3 protein bands were normalized to β-actin. The data are representative of three experiments.

Fig. 7, the transcript level of *PFKFB* genes in gastric non-malignant tissue was maximal for *PFKFB*-2, slightly less for *PFKFB*-3 and minimal for *PFKFB*-1 and *PFKFB*-4. Analysis of the expression of *PFKFB*-1, *PFKFB*-2, *PFKFB*-3 and *PFKFB*-4 mRNA in malignant gastric tumors showed that expression of all these mRNAs was increased in the tumor but the strongest induction was observed for *PFKFB*-3 mRNA. The expression of known HIF-1-dependent genes *Glut1* and *VEGF* was also increased in gastric malignant tumors.

Using Western blotting we measured the protein levels of PFKFB-4 and PFKFB-3 as well as the levels of the inducible isoform of the PFKFB-3 isozyme. As shown in Fig. 8, the level of PFKFB-4 protein expression in gastric cancers was much higher as compared to the PFKFB-3 isoenzyme. The expression of PFKFB-4, PFKFB-3 and iPFKFB-3 proteins was increased in malignant gastric tumors as compared to corresponding nonmalignant tissue. Thus, the main protein isoform of PFKFB expressed in both gastric malignant tumors and nonmalignant tissue is PFKFB-4.

DISCUSSION

This study clearly demonstrates that 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 mRNA is expressed in the pancreatic cancer cell line Panc1 and in two gastric cancer cell lines MKN45 and NUGC3 and that both genes are responsive to hypoxia *in vitro*. The ribonuclease protection analysis also showed that *PFKFB*-4 and *PFKFB*-3 mRNA expression in both gastric and pancreatic cancer cell lines is highly responsive to dimethyloxalylglycine in normoxic conditions. However, the basal transcript level of *PFKFB*-4 and *PFKFB*-3 and their hypoxia responsiveness vary in the different cell lines studied. Moreover, no strong correlation is present between *PFKFB*-3 or *PFKFB*-4 mRNA and protein expression in the gastric and pancreatic cancer cells, both in normoxic and hypoxic conditions. This data concurs with our previous results (Minchenko *et al.*, 2002; 2004; 2005c).

Thus, we have previously shown that hypoxia strongly induces the expression of *PFKFB*-3 and *PFKFB*-4 genes in many cancer cell lines *via* HIF-dependent mechanism in a cell-specific manner. Moreover, the analysis of *PFKFB*-4 transcript and protein levels in different mammary gland malignant cell lines clearly demonstrated that no strong correlation is present between *PFKFB*4 mRNA and protein expression, both in normoxic and hypoxic conditions (Minchenko *et al.*, 2005c). Thus, the basal and hypoxia-inducible expression of *PFKFB*-4 mRNA is higher in the T47D mammary gland adenocarcinoma cell line as compared the MCF7 mammary gland adenocarcinoma cell line. However, PFKFB-4 protein expression is higher in the MCF7 cell line as compared to T47D cells, both in normoxic and hypoxic conditions (Minchenko *et al.*, 2005c). Moreover, SKBR3 and MDA-MB-468 mammary gland adenocarcinoma cell lines have similar *PFKFB*-4 mRNA expression both in normoxic and hypoxic conditions. Unexpectedly, no significant levels of PFKFB4 protein were observed in the MDA-MB-468 cancer cell line in the experimental conditions used for SKBR3 or other cell lines. In contrast, the basal and hypoxia-inducible expression of *PFKFB*-4 mRNA is correlated with PFKFB-4 protein level in the SKBR3 and BT549 mammary gland adenocarcinoma cell lines (Minchenko *et al.*, 2005c). The results of this study demonstrate a similar basal expression of *PFKFB*-4 mRNA and a significantly different basal expression of PFKFB-4 protein in gastric and pancreatic cancer cell lines. Thus, the basal level of PFKFB-4 protein is the lowest in the Panc1 cell line and highest in NUGC3 gastric cancer cells. The level of hypoxic regulation of *PFKFB*-4 mRNA and protein varies in different gastric cancer cell lines as well as in the Panc1 cell line.

It is interesting to note that there is an inverse correlation between the basal levels of PFKFB-4 protein expression and the hypoxic responsiveness of *PFKFB*-4 mRNA expression, although its biological significance remains to be determined. Indeed, the lowest basal level of PFKFB-4 protein, which is observed in the pancreatic cancer cell line Panc1, is correlated with the highest hypoxic induction of *PFKFB*-4 mRNA and protein expression; however, the hypoxia-induced level of PFKFB-4 protein in these cells is in fact lower when compared to the basal level of PFKFB-4 protein expression in NUGC3 cells. Moreover, the highest basal level of PFKFB-4 protein, which we observed in the gastric cancer cell line NUGC3, is correlated with the lowest hypoxic induction of *PFKFB*-4 mRNA and protein expression; on the other hand, the hypoxia-induced level of PFKFB-4 protein in these cells is very high as compared to the basal or hypoxia-induced levels of PFKFB-4 protein in both Panc1 and MKN45 cells. Thus, the results of Western blot analysis with specific anti-human PFKFB-4 antibodies revealed that the constitutive levels of PFKFB-4 protein in the MKN45 and NUGC3 gastric cancer cell lines as well as in the Panc1 pancreatic cancer cell line are different and are increased by hypoxia. These observations suggest that the increase in PFKFB-4 protein expression was not reflected at the mRNA level. It is possible that this discrepancy between *PFKFB*-4 mRNA and protein levels, which were found in the different gastric and pancreatic cancer cell lines, is related to the mechanism which controls stability of PFKFB-4 protein. However, the precise molecular mechanism for these inverse correlations is complex and possibly includes post-translational modification and stability of PFKFB-4 enzyme in a cell type-specific manner and warrants further investigation.

The induction of *PFKFB*-3 mRNA expression in the NUGC3 gastric cancer cell line by hypoxia and dimethyloxalylglycine is much stronger as compared to *PFKFB*-4 mRNA expression. It is important to note that *PFKFB*-3 gene in the Panc1 pancreatic cancer cells has the lowest hypoxia responsiveness as compared to both gastric cancer cell lines. We have previously shown that the hypoxic induction of *PFKFB*-3 mRNA expression in mammary gland cancer cells is much stronger in MCF7 and T47D breast cancer cells (estrogen receptor-positive cell lines) as compared to SKBR-3 and MDA-MB-468 cells (estrogen receptor-negative cell lines) (Minchenko *et al.*, 2005c). The different sensitivity of the PFKFB-3 isoenzyme to hypoxic induction was shown recently for many other cell lines (HeLa, Hep3B, RPE, and fibroblasts), while hypoxic induction of Glut1 gene was similar in these cell lines (Minchenko *et al.*, 2002).

The results of this investigation provide clear evidence that the hypoxic induction of *PFKFB*-4 gene expression was replicated by dimethyloxalylglycine in the different gastric and pancreatic cancer cell lines, suggesting that the hypoxia responsiveness of this gene is regulated by HIF proteins. Dimethyloxalylglycine (an oxoglutarate analog) is a specific inhibitor of prolyl hydroxylases, protects the HIF-1α protein from proteasomal degradation and significantly increases its level (Epstein *et al.*, 2001; Schofield & Ratcliffe, 2004). Inhibition of these enzymes can induce the levels and transcriptional activity of HIF-1α under normoxic conditions and mimics hypoxic conditions (Metzen & Ratcliffe, 2002). Thus, using dimethyloxalylglycine we can identify the HIF-dependent effects of hypoxia.

Recently was shown that hypoxic induction of *PFKFB*-4 gene transcription is mediated by the hypoxia responsive element located in the 5′-promoter region of the human *PFKFB*-4 gene (293–300 bp upstream from the GATA site) and that this hypoxia responsive element has homology with the same described in other hypoxia responsive genes (Gleade & Ratcliffe, 1998; Minchenko & Caro, 2000; Wenger, 2002; Fukasawa *et al.*, 2004; Minchenko *et al.*, 2005c). Recently the hypoxia responsive element was identified in the promoter region of *PFKFB*-3 gene which mediates hypoxia responsiveness on the transcriptional level (Fukasawa *et al.*, 2004; Obach *et al.*, 2004).

The expression of *VEGF* and *Glut1*, which are known HIF-dependent genes, is also strongly induced under hypoxic conditions in gastric and pancreatic cancer cell lines. The significant induction of HIF-1α protein in both gastric and pancreatic cancer cell lines under hypoxic conditions supports the HIF-1α-dependent character of the induction of expression of the genes studied. However, the basal level of *HIF*-1α as well as *HIF*-2α mRNA expression and their hypoxia responsiveness is different in the MKN45 and NUGC3 cancer cells. Thus, the expression of *HIF*-1α mRNA is decreased in both gastric cancer cell lines treated by hypoxia or dimethyloxalylglycine, but no significant changes of *HIF*-2α mRNA expression were found in the NUGC3 gastric cancer cell line under hypoxia. However, the expression of *HIF*-2α mRNA in the MKN45 gastric cancer cell line was slightly induced by dimethyloxalylglycine and hypoxia. A similar pattern of *HIF*-1α and *HIF*-2α mRNA expression in hypoxic conditions was shown in the A_{549} lung adenocarcinoma cell line (Uchida *et al.*, 2004). Importantly, there is an inverse correlation between the hypoxic responsiveness of HIF-1α mRNA and protein expression. These observations suggest that the increase in HIF-1α protein expression was not reflected at the mRNA level. Moreover, the expression of *HIF*-1α mRNA is significantly decreased both under hypoxic condition and by dimethyloxalylglycine action. It is possible that this discrepancy between *HIF*-1α mRNA and protein levels, which was found in different gastric and pancreatic cancer cell lines, is related to the divergence in mechanisms which control the stability of *HIF*-1α mRNA and protein. Thus, the hypoxic induction of HIF-1α protein expression is a result of its stabilization which is mediated by specific oxygen- and iron-dependent prolyl hydroxylase enzymes that utilize oxoglutarate as a co-substrate (Epstein *et al.*, 2001; Lando *et al.*, 2002; Mole *et al.*, 2003; Schofield *et al.*, 2004). There is data that hypoxia and dimethyloxalylglycine decrease HIF-1α mRNA levels in a cell type-specific manner (Minchenko & Caro, 2000; Marti *et al.*, 2002; Uchida *et al.*, 2004; Minchenko *et al.*, 2006). The dimethyloxalylglycine responsiveness of *HIF*-1α mRNA expression suggests that *HIF*-1α protein can mediate *HIF*-1α mRNA expression during hypoxia. Uchida *et al.* (2004) have shown that hypoxia induce HIF-1α protein and reduce *HIF*-1α mRNA expression in A_{549} lung carcinoma cell line, but *HIF*-2α mRNA is slightly increased. This data is closely correlated with the results of the present investigation: hypoxia reduces HIF-1α and slightly increases *HIF*-2α mRNA expression in the gastric cancer cell line MKN45. This effect is cell-specific because it is not observed in the NUGC3 gastric cancer cell line.

Cancer cells show elevated glycolytic rates, produce high levels of lactate and pyruvate (the Warburg effect), and this correlates with an increased expression of glycolytic enzymes and glucose transporters *via* a HIF-dependent mechanism (Lu *et al.*, 2002; Hopfl *et al.*, 2004). Since PFKFB isoenzymes catalyze the synthesis and degradation of fructose-2,6-bisphosphate, they control glycolysis and play a significant role in the Warburg effect, which is typical for tumor cells (Chesney *et al.*, 1999; Minchenko *et al.*, 2002; 2004; Rider *et al.*, 2004). The major finding reported here is that several isoenzymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase with different kinetics and regulatory properties are overexpressed in solid gastric tumors as compared to nonmalignant tissue counterparts from the same patients, suggesting that all four isoenzymes may contribute to the Warburg effect. Our study provides evidence that the *PFKFB*-4 gene is overexpressed in most cancer cells, is strongly responsive to hypoxia, and may have a significant role in the Warburg effect, much like *PFKFB*-3. Recently, we have shown that lung cancers also overexpress all of the *PFKFB* genes (Minchenko *et al.*, 2005b). Of interest is that the level of different *PFKFB* gene expression differs in lung and gastric cancers and nonmalignant tissues. These observations suggest that different *PFKFB* genes are expressed in nonmalignant tissues and cancers in a cell-specific manner. Thus, in lung cancers expression of *PFKFB*-4 and *PFKFB*-3 mRNA is increased much more strongly than in gastric cancers as compared to nonmalignant tissue counterparts (Minchenko *et al.*, 2005b).

Moreover, this study provides evidence that the high level of PFKFB-4 protein expression was detected in gastric malignant tumors. Similar results were shown in the colon, breast and lung cancers (Minchenko *et al.*, 2005a; 2005b). However, the level of PFKFB-4 protein in the lung and breast nonmalignant tissues is very low in comparison to the gastric normal tissues, but its expression much higher in the lung and breast malignant tumors, as opposed to the gastric and colon cancers (Minchenko *et al.*, 2005a; 2005b). It is interesting to note that there is an inverse correlation between the levels of PFKFB-4 protein expression in normal (nonmalignant) tissues and its induction in cancers: induction of PFKFB-4 protein expression is much stronger in malignant tumors from organs which have low levels of PFKFB-4 protein in nonmalignant tissues.

There is data that the PFKFB-3 isoenzyme is highly expressed in transformed cells and malignant tissues, suggesting that it may contribute to the high glycolytic rate observed in tumors (Chesney *et al.*, 1999; Atsumi *et al.*, 2002; Minchenko *et al.*, 2005a; 2005b). Interestingly, the level of PFKFB-4 protein in gastric, lung, breast and colon cancers is much higher as compared to the PFKFB-3 isoenzyme. Thus, the PFKFB-4 isoenzyme should be considered a tumor-specific enzyme in the lung, breast, gastric and colon cancers and may also contribute to the high glycolytic rate observed in tumors, much like PFKFB-3. There is also data that a molecule of PFKFB-3 in which EDTA is covalently linked to ADP is a good starting molecule for the development of new cancer-therapeutic molecules (Kim *et al.*, 2006). It is important to note that most isoenzymes of the *PFKFB* gene family may find clinical utility as novel targets for the development of new anticancer agents.

In conclusion, this study provides evidence that *PFKFB*-4 and *PFKFB*-3 genes are expressed in gastric and pancreatic cancer cells and strongly respond to hypoxia *via* a HIF-1α dependent mechanism but no strong correlation is present between *PFKFB*-3 or *PFKFB*-4 mRNA and protein expression in these cancer cells, both in normoxic and hypoxic conditions. Moreover, there is an inverse correlation between the basal levels of PFKFB-4 protein expression and the hypoxic responsiveness of *PFKFB*-4 mRNA expression, although the biological significance of this is currently unknown and warrants further investigations. Hypoxic induction of HIF-1α protein in these cell lines correlates with a reduction of HIF-1α mRNA expression.

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