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Examination of Intra-Tumoral Expression of the PFKFB3 and PFKFB4 Enzymes: Implications for Targeting Glycolysis

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Abstract

A high rate of glycolytic activity is observed in multiple tumor types and serves to provide energy and biosynthetic precursors to support their growth and spread. The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family of enzymes (PFKFB1-4) have been recognized as important regulators of glycolysis due to their production of fructose-2,6-bisphosphate (F26BP) which activates a rate-limiting glycolytic enzyme, 6-phosphofructo-1-kinase (PFK-1). As a result, the expression and function of the PFKFB enzymes have been extensively evaluated. In these studies, we found that the PFKFB3 and PFKFB4 enzymes were consistently co-expressed in multiple tumor tissues. Further evaluation of their expression showed that PFKFB3 and PFKFB4 were similarly expressed in normoxia but exhibited significant expression differences under hypoxic conditions where PFKFB4 was highly induced. We additionally found that the isoforms showed different intratumoral expression wherein PFKFB4 correlated significantly with areas of hypoxia and PFKFB3 showed poor correlation. Based on their varied expression, we examined the effects of silencing both isoforms and found that co-silencing PFKFB3 and PFKFB4 significantly decreased proliferation, particularly in hypoxia. These results, although preliminary, carry implications for the potential utility of targeting both the PFKFB3 and PFKFB4 enzymes for the development of effective therapeutic approaches against cancer.

Keywords: Glycolysis; Hypoxia; PFKFB; PFKFB3; PFKFB4; Cancer

Introduction

Position emission tomographic studies using ^{18}F-fluoro-deoxy-glucose (FDG-PET) consistently demonstrate significantly higher glucose uptake in multiple tumor types relative to adjacent normal tissues [1]. Glucose taken up by tumors is utilized to generate energy and biosynthetic intermediates through glycolysis to support their rapid growth and metastasis [2]. A metabolic shift to a high rate of glycolysis is demonstrated by the majority of tumors and is widely held to be a hallmark of cancer [3].

The first committed step in the glycolytic pathway is the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F16BP) catalyzed by the 6-phosphofructo-1-kinase enzyme (PFK-1) which therefore plays a critical and rate-limiting role in glycolysis [4,5]. The activity of PFK-1 is regulated by several metabolites of which the molecule fructose-2,6-bisphosphate (F26BP) has been determined to be the most powerful activator [6-8]. F26BP is produced by the kinase activity of a family of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) enzymes which interconvert F6P and F26BP [9]. Accordingly, the PFKFB enzymes

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play a key regulatory role in glycolysis and have been the subject of significant scrutiny.

The PFKFB family of enzymes consists of four members (PFKFB1-4) that share high sequence homology (>85%) in their core catalytic domain but exhibit differences in their N and C-terminal domains. As a result, they show variations in their kinase and bisphosphatase activities and kinase:bisphosphatase (K:B) ratios which result in distinct differences in their relative contributions to cellular F26BP levels and to the regulation of glycolysis [6,9,10]. Of the 4 isoforms, the highest kinase activity is exhibited by the PFKFB3 enzyme (with a K:B ratio of ~740:1) followed by the PFKFB4 enzyme which has a K:B ratio of 4.3:1 [11-14]. The PFKFB2 and PFKFB1 enzymes have lower ratios of ~1.8:1 and ~1:1 [11,12] respectively [11,13]. In efforts to determine the contribution of the different PFKFB isoforms to intracellular F26BP levels and glycolytic regulation, previous studies from our laboratory evaluated the expression of the four PFKFB isoforms in a variety of tumor cell lines and in patient-derived matched normal and tumor tissue using multiplex PCR [12,15]. We found that the majority of tumor types showed the simultaneous expression of several PFKFB isoforms. Based on their high kinase activity, we hypothesized that the PFKFB3 and PFKFB4 enzymes are likely responsible for maintenance of intracellular F26BP concentrations in cancer cells and tumors and therefore critical for regulating tumor glycolysis. Importantly, with the development of effective small molecule inhibitors targeting the PFKFB3 and PFKFB4 enzymes, the identification of the isoform or isoforms that play a dominant role in regulation will allow the development of effective anti-neoplastic therapeutic strategies [16-18].

To that end, we first assessed PFKFB1-4 expression in cell lines derived from multiple cancer types and found that PFKFB3 and PFKFB4 were co-expressed in all lines screened. Since both enzymes have high kinase activity and have been shown to be induced by hypoxia [19], we sought to determine their relative contribution to tumor glycolytic regulation in conditions of normoxia and, importantly, in hypoxia, given the high prevalence of hypoxic conditions in rapidly growing tumors and their reliance on glycolysis to support proliferation under hypoxia. In these studies, we confirmed that PFKFB3 and PFKFB4 are expressed and contribute to proliferation in rapidly growing tumors but found significant variations in their expression, particularly under hypoxic conditions. Our results indicate that co-expression of the PFKFB3 and PFKFB4 isoforms may form an important aspect of the tumor survival mechanism and provide rationale for the examination of combined targeting of PFKFB3 and PFKFB4 as a potential therapeutic option against cancer.

Materials and Methods

Cell lines and treatments

A549 and H460 human lung adenocarcinoma, Colo320 human colon adenocarcinoma and HL60 and K562 human leukemia cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in DMEM or RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% fetal calf serum (FCS, Life Technologies) in a 37ºC incubator in 5% carbon dioxide (CO_2). For siRNA experiments, cells growing in 6-well plates were transfected with control (siCtrl, Stealth Negative Control, Invitrogen, Carlsbad, CA), PFKFB3 (siFB3; HSS107860, Invitrogen) or PFKFB4 (siFB4, HSS107863, Invitrogen) siRNA using Lipofectamine RNAiMax (Invitrogen), and harvested 48 hours after transfection. For hypoxia experiments, cells were exposed to hypoxia for indicated periods of time or were transfected with siRNA and, after 24 hours, placed in hypoxia. Hypoxia was achieved by use of a hypoxia chamber (Billups-Rothenburg, Del Mar, CA) purged with a gas mixture of 1% oxygen, 5% CO_2 and 94% nitrogen.

Assessment of cell viability and counts

Cell viability and proliferation were assessed using Trypan blue exclusion as previously described [17]. Cells for counting were detached using 0.25% Trypsin EDTA (Life Technologies) neutralized with complete media and were incubated in 20% Trypan blue (Sigma, St. Louis, MO) for 5 minutes. Cells excluding Trypan blue were counted using a standard hemocytometer (Hausser Scientific) to determine total numbers of viable cells. Data are expressed as mean ± SD of three experiments. Statistical significance was assessed by the two-sample t test (independent variable).

PCR analyses

Multiplex mRNA primers were custom synthesized (IDT, Coralville, IA) against human PFKFB1-4 as previously described [15]. cDNA synthesized from indicated human cancer cell lines was analyzed using these primers under standard PCR conditions. PFKFB3 and 4 mRNA expression was determined using real-time RT-PCR with TaqMan probes for human PFKFB3, PFKFB4 and β-actin (Applied Biosystems, Foster City, CA) in triplicate in 96-well optical plates (MicroAMP®, Applied Biosystems). Analysis of results and fold differences between samples were determined using StepOne software (Applied Biosystems) and calculated from ΔΔCT values with the formula (2-ΔΔCT). The data are represented as the mean ± SD from triplicate measurements from three independent experiments. Statistical significance was assessed by the two-sample t test (independent variable).

Protein extraction and Western blotting

Cells, following treatment as indicated above, were harvested, washed in PBS and lysed in 1X lysis buffer (Pierce

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Biotechnology, Rockford, IL) containing protease inhibitors. Protein samples were resolved on 4-15% SDS-PAGE gels and transferred to PVDF membranes (both from BioRad, Hercules, CA). After blocking in TBS containing 0.1% Tween 20 (TBS-T) and 5% milk, membranes were probed with antibodies to PFKFB3 (Proteintech, Chicago, IL), PFKFB4 (Epitomics, Burlingame, CA) or β-actin (Sigma). Secondary antibodies used were HRP-conjugated goat anti-rabbit or anti-mouse (Pierce Biotechnology, Waltham, MA). Scanned images were quantified by densitometric analyses using Image J software (http://rsb.info.nih.gov/ij/). Values obtained were normalized to β-actin. The data represented are the mean ± SD from triplicate measurements from three independent experiments. Statistical significance was assessed by the two-sample t test (independent variable).

Immunohistochemistry

Five µm mounted sections of formalin-fixed and paraffin-embedded tumor tissue were deparaffinized with xylene. Epitope retrieval was carried out using citrate buffer in a 2100 Retriever (Aptum Biologics, Southampton, Hants, UK). The sections were blocked with 10% goat serum for 1 hour, then incubated with primary antibodies against PFKFB3, PFKFB4 or carbonic anhydrase IX (CA IX, Proteintech) overnight, followed by an HRP-linked goat anti-rabbit secondary antibody (Pierce Biotechnology). The sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Burlingame, CA) for 2 min, nuclei counterstained with Mayer's hematoxylin (Sigma) for 2 min and coverslips attached with Permount (Fisher Scientific, Fair Lawn, NJ). Slides were scanned using a ScanScope XT Digital Slide Scanner (Aperio), data analyzed with the positive pixel count algorithm (ImageScope, Aperio) as the % positive pixels/total pixels ± SD with a minimum of 5 fields (20x magnification) quantified for each tumor section. GraphPad Prism 7 software (Boston, MA) was used for linear regression analyses.

Results

The PFKFB3 and PFKFB4 enzymes are co-expressed in multiple types of cancer

Previous data from our laboratory have demonstrated that mammalian tissues express multiple PFKFB isoforms simultaneously [15]. We examined cDNA derived from cells from varied types of cancer by multiplex PCR and confirmed that multiple PFKFB enzymes were frequently expressed simultaneously and found that, of the PFKFBs, the PFKFB3 and PFKFB4 enzymes showed the highest expression in the majority of cell lines examined, in addition to co-expression (Figure 1, A).

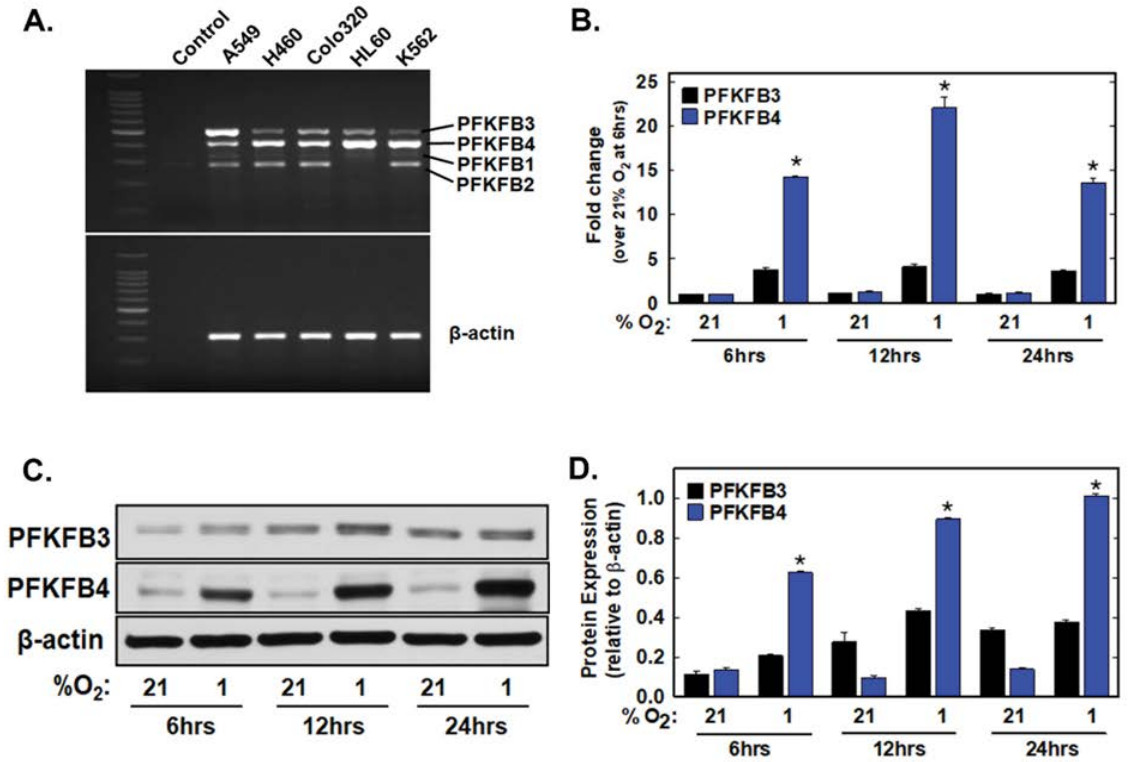

Figure 1: The PFKFB3 and PFKFB4 enzymes demonstrate varied levels of induction by hypoxia *in vitro*. PFKFB1-4 expression in indicated cell lines was assessed by multiplex RT-PCR, β-actin as control (A). H460 cells were exposed to 21 or 1% oxygen for indicated periods of time and PFKFB3 and PFKFB4 mRNA expression was assessed by real-time RT-PCR (B), protein expression was assessed by Western blot (C) and was quantitated by densitometry (D). * *p* value <0.05.

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PFKFB4 expression is significantly induced by hypoxia in tumor cells *in vitro*

Both the PFKFB3 and PFKFB4 enzymes demonstrate hypoxia response elements in their 5' promoter regions and their expression is observed to be induced by exposure to a low oxygen concentration as is frequently found in rapidly growing tumors [20]. Since PFKFB3 and PFKFB4 are simultaneously expressed in multiple cancer cell lines, we sought to compare their response to hypoxic conditions to determine if variations in their responses might indicate differences in the role of these enzymes. We selected a lung cancer cell line that co-expresses PFKFB3 and PFKFB4 (H460 lung adenocarcinoma cells) for our examination and exposed H460 cells to 21 or 1% oxygen over a 24 hour period. We found that the enzymes showed dissimilar responses to hypoxic exposure (Figure 1, B-D). We observed a significant induction of PFKFB4 expression in hypoxia relative to normoxia over time and, in contrast, found that PFKFB3 expression was similar at all time points examined with a slight increase in expression demonstrated at 12 and 24 hours of exposure relative to its normoxic level of expression (Figure 1, B-D).

PFKFB4 expression shows significant correlation with areas of tumor hypoxia *in vivo*

The growth of tumors is associated with areas of hypoxia resulting from their rapid proliferation and disorganized blood vessel development [20]. Previous data from our laboratory have found that PFKFB4 expression showed a strong correlation with areas of hypoxia in tumors *in vivo* [12]. Based on the differences that we observed in PFKFB3 and PFKFB4 induction from exposure to hypoxia *in vitro*, we next wanted to compare the expression of these enzymes in the entire tumor *in vivo* and to correlate their expression with hypoxic areas using carbonic anhydrase IX (CA IX), which is a transcriptional target of HIF-1α and strongly induced by its activity [21]. We examined serial sections of entire xenograft tumors harvested from mice for expression of CA IX, PFKFB3 and PFKFB4 (Figure 2, A-F) and then correlated PFKFB3 and PFKFB4 expression with the expression of CA IX. Similar to our previous data, we found that PFKFB4 expression correlated significantly with CA IX expression but found that, in line with our *in vitro* findings, PFKFB3 was expressed at low levels in the tumor sections without significant correlation to CA IX expression (Figure 2, G and H).

Simultaneous silencing of PFKFB3 and PFKFB4 in hypoxia significantly decreases cell proliferation

Based on the observed intra-tumoral co-expression of PFKFB3 and PFKFB4, we hypothesized that both isoforms might be required to regulate glycolysis and support cancer cell proliferation and survival. To examine the requirement for

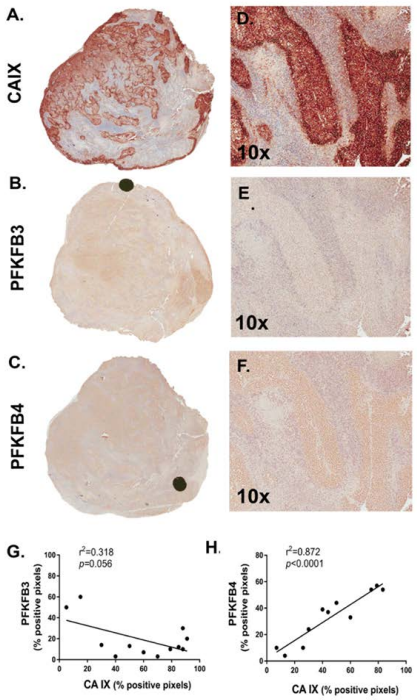

Figure 2: PFKFB4 expression correlates with hypoxic regions in xenograft tumors *in vivo*. Lung tumors (H460) were analyzed by immunohistochemistry for CA IX, PFKFB3 and PFKFB4 expression (**A-C**, 2x magnification; **D-F**, 10x). Images were scanned and positive pixels enumerated in a minimum of 5 fields per tumor section followed by linear regression analysis (**G, H**). r^2 and *p* values are provided.

PFKFB3 and/or PFKFB4 in cancer cell growth, we silenced their expression using siRNA (that were previously validated in this cancer cell line[12]) and examined cell proliferation and viability over 48 hours in the presence of either 21 or 1% oxygen. We found that silencing either isoform decreased cell proliferation significantly under normoxic conditions and observed that under hypoxia, silencing PFKFB4 expression led to a greater suppressive effect on cell proliferation (Figure 3). We also found that simultaneous silencing of PFKFB3 and PFKFB4 caused a greater decrease in cell proliferation than silencing either isoform individually (Figure 3).

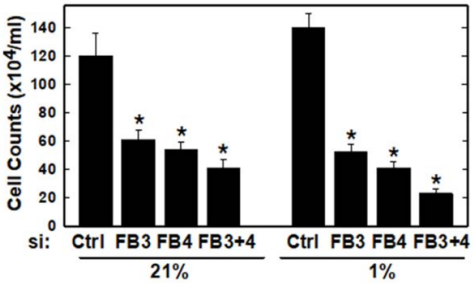

Figure 3: Simultaneous silencing of PFKFB3 and PFKFB4 in hypoxia significantly decreases cell proliferation. H460 cells were transfected with indicated siRNA and exposed to 21 or 1% oxygen. Viable cells were counted 48 hours after transfection. **p* value <0.05.

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Discussion

The reprogramming of metabolism by tumors to increase their dependence on glycolysis is a key hallmark of cancer [3]. Tumors rely on glycolysis to fuel their rapid proliferation and spread [2]. Due to the growing recognition of the importance of glycolytic metabolism in supporting tumor growth, its regulation is an area of active investigation which includes numerous studies that have examined the role of the PFKFB family of enzymes in this process [18]. Due to the production of F26BP through their kinase activity, the PFKFB enzymes regulate a key rate-limiting step of glycolysis and the interrogation of their functions will allow the development of strategies to limit tumor glycolysis and thereby decrease tumor growth [9].

Our studies have found that the PFKFB3 and PFKFB4 isoforms are consistently co-expressed in a variety of solid and hematogenous tumors. Of the PFKFB family members, PFKFB3 and PFKFB4 have the highest K:B ratios and are therefore responsible for the greatest contribution to intracellular F26BP levels and thereby to glycolytic regulation [11-14]. The expression of both PFKFB3 and PFKFB4 enzymes has been observed to be induced by hypoxia (likely through the transcription factor HIF-1α) and both enzymes also express hypoxia response elements in their 5' promotor regions [19,22,23]. Since rapid tumor growth is known to be accompanied by significant areas of intra-tumoral hypoxia [20], a goal of our studies was to compare the responses of these two isoforms to hypoxia. Our preliminary evidence demonstrates that the PFKFB3 and PFKFB4 enzymes are induced to different levels in response to hypoxia. We found that expression of PFKFB3 was largely unchanged by exposure to hypoxia *in vitro* and showed similar expression in normoxia and hypoxia over the time period evaluated. In contrast, we found that PFKFB4 expression was highly induced by hypoxia *in vitro* and that its expression was significantly increased in hypoxia relative to normoxic levels and also increased over the evaluated time period. Due to the consistent expression of PFKFB3 and its higher kinase activity, we surmise that its contribution to F26BP levels is substantially higher relative to PFKFB4 in normoxic conditions. In contrast, with its high induction of expression in hypoxia, we postulated that PFKFB4 may contribute more significantly to F26BP levels in hypoxic conditions. Studies to examine the effects of hypoxic induction of both enzymes on glycolysis are ongoing.

To investigate the responses of the PFKFB3 and PFKFB4 enzymes to hypoxia in the more relevant setting of *in vivo* tumor growth, we next examined PFKFB3 and PFKFB4 expression in serial sections taken from xenograft tumors. To determine the correlation of their expression with areas of hypoxia, we also examined consecutive sections of these tumors for CA IX, a transcriptional target of HIF-1α [21]. We found, similar to our *in vitro* findings, that PFKFB3 was expressed over multiple areas of the tumors but poorly correlated to CA IX expression and that, in contrast, PFKFB4 expression correlated very closely with areas of tumor hypoxia. Based on these preliminary data, we hypothesize that expression of both isoforms may be critical in the regulation of intra-tumoral glycolysis, with PFKFB3 relevant in normoxic areas of the tumors and PFKFB4 serving as a key component of the tumor hypoxic response that permits cancer cell survival and proliferation in hypoxic conditions. Most importantly, based on these data, we examined the effects of silencing either or both PFKFB3 and PFKFB4 simultaneously and found that simultaneous silencing of both enzymes significantly decreased tumor cell proliferation and survival.

The importance of glycolysis in the growth and metastasis of tumors has led to multiple efforts to target the glycolytic pathway with inhibitors for the treatment of cancer but none of these efforts have yielded successful anti-cancer agents for clinical use in patients [24]. Given the critical role played by glycolysis, targeting the regulation of the glycolytic pathway holds promise and several small molecule inhibitors targeted against PFKFB3 and PFKFB4 have been identified. These agents are currently under active pre-clinical and clinical development and have been shown to significantly decrease tumor glycolysis and growth without any noted toxicity [16-18]. The results of our studies confirm the expression and importance of the PFKFB3 and PFKFB4 enzymes in cancer cell growth and proliferation. Although we fully recognize that these studies are highly preliminary and require further evaluation of the expression differences of PFKFB3 and PFKFB4 in normoxia and hypoxia and the resultant changes in their effects on metabolism, they carry significant relevance for the development of novel therapeutic strategies using agents to simultaneously inhibit both enzymes. Studies to evaluate the effects of these inhibitors in combination strategies are currently underway in our laboratory with the goal of developing effective therapeutic options against cancer.

Conclusion

In conclusion, our data demonstrate that the glyco-regulatory enzymes PFKFB3 and PFKFB4 are consistently co-expressed in cancer cells *in vitro* and in tumors *in vivo* and further that, of the two enzymes, PFKFB4 is significantly induced by hypoxia and correlated with tumor hypoxic areas *in vivo*. Additionally, our data show that the simultaneous silencing of the PFKFB3 and PFKFB4 enzymes decreases proliferation of cancer cells *in vitro*. These studies provide preliminary evidence that simultaneous targeting of the PFKFB3 and PFKFB4 enzymes may serve as a novel and effective targeting strategy against cancer.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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