Phosphoglucose isomerase enhances colorectal cancer metastasis

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Abstract. Phosphoglucose isomerase (PGI) is a ubiquitous cytosolic enzyme that plays a key role in glycolysis. PGI is also a multifunctional protein that acts in the extracellular milieu as a potent mitogen/cytokine. Increased expression of PGI and its receptor has been found in a wide spectrum of malignancies and is associated with cancer progression and metastasis. In this study, the role of PGI in the growth and metastasis of colon cancer cells was determined. To elucidate the functional role of PGI in colorectal cancer, we stably transfected PGI cDNA into human colon cancer cells. We used an orthotopic mouse tumor model to assess whether overexpression of PGI enhances liver metastasis. Overexpression of PGI stimulated the *in vitro* invasion of DLD-1 cells. *In vivo*, after orthotopic implantation into the cecum of nude mice, parental and empty vector-transfected DLD-1 cells produced small tumors without liver metastasis, whereas PGI-overexpressing DLD-1 cells produced large tumors and liver metastases. In conclusion, overexpression of PGI significantly contributes to the aggressive phenotype of human colon cancer and, thus, may provide a novel therapeutic target.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer-related death in both men and women in the US, with an estimated 49,960 deaths in 2008 (1). Approximately 30% of all patients with CRC have metastatic disease at diagnosis, and 50% of early stage patients eventually develop metastatic or advanced disease (2). The introduction of new chemotherapeutic regimens, including FOLFOX + bevacizumab and FOLFIRI + bevacizumab, has increased the median survival of these patients (3). However, the results of chemotherapy remain inferior to those of curative hepatic resection, which results in 5-year survival rates of 40 to 50% in selected patients (4,5). More effective modalities of treatment for metastatic CRC are needed.

Cancerous tissue exhibits a significantly higher glucose consumption than normal tissue. Moreover, a higher glucose uptake correlates with higher tumor aggressiveness and progressively poorer prognosis. Cancer cells maintain high aerobic glycolytic rates and produce high levels of lactate and pyruvate. Aerobic glycolysis in tumors can be regulated at the level of glycolytic enzymes or glucose transporters in tumor cells. Phosphoglucose isomerase (PGI; EC 5.3.1.9) is a cytosolic enzyme that plays a key role in both glycolysis and gluconeogenesis pathways (6). Molecular cloning and sequencing have identified PGI with the autocrine motility factor (AMF). AMF is a malignant factor that enhances the invasive and metastatic abilities of tumor cells (7,8). AMF is an orphan CXXC chemokine, and its cognate receptor is a unique seven-transmembrane glycoprotein receptor (autocrine motility factor receptor, AMFR). Overexpression of AMF/PGI and AMFR has been found in a number of different malignancies and is associated with cancer progression and metastasis (7,9,10). In this study, we examined whether PGI contributes to the distant metastasis of CRC.

Materials and methods

Materials. The following antibodies were used. Anti-ß-actin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-AMF and anti-AMFR were described previously (7). We purified anti-AMF IgG and preimmune IgG from serum using ImmunoPure (G) IgG according to the manufacturer's instructions (Pierce, Rockford, IL).

Cell culture and transfection. DLD-1 human colon carcinomas were purchased from the Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), essential and nonessential amino acids, and penicillin/streptomycin. The cells were maintained in a humidified chamber with 95% air and 5% CO_2 at 37˚C.

The full-length human PGI cDNA was generated by PCR amplification (11). The PCR product was ligated into a mammalian expression vector pcDNA3.1 zeo (Invitrogen, Carlsbad, CA). According to the manufacturer's instructions, Lipofectamine 2000 (Life Technologies, Inc., Gaithersburg,

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MD) was used to transfect parental DLD-1 cells with PGI cDNA. Isolation of single clones of the stable transfectants was accomplished by adding 750 μg/ml of Zeocin (Invitrogen) to the culture medium. The DLD-1 cell line, stably transfected with pcDNA3.1 zeo or pcDNA3.1 zeo-PGI, was designated DLD-zeo or DLD-PGI, respectively. All experiments were repeated at least three times, and results were confirmed by both clonal cell lines and the pooled cell population.

Western blot analysis. The cells were lysed in a lysis buffer [50 mmol/l HEPES (pH 7.9), 0.1% NP40, 10% glycerol, 1 mmol/l DTT, 1% sodium deoxycholate, 250 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride and 0.1 mg/ml leupeptin] at 4˚C. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (MSI, Westborough, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T), incubated with the primary antibody for 2 h, washed 3 times within 15 min in TBS-T, incubated with the secondary horseradish peroxidase-conjugated antibody (Zymed, San Francisco, CA) for 1 h, and finally washed three times. The horseradish peroxidase activity was detected by incubation of the membrane with an enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL). A Kodak imaging system was used to determine the density of the bands.

Cell proliferation assays. Cell proliferation assays were conducted by seeding cells at a density of $1x10^5$ cells/well in 6-well plates. Cells were fed RPMI-1640 with 10% FBS every other day and counted daily.

Invasion assay. The invasive activity of cells was assayed in transwell cell culture chambers (Corning Costar Co., Cambridge, MA). Polycarbonate filters with an 8-μm pore size were coated with Matrigel (1 mg/ml) (Collaborative Biomedical Products, Bedford, MA) to form a matrix barrier. Cells were resuspended to a concentration of $1x10^6$/ml in RPMI-1640 with 0.5% FBS. The cell suspension (100 μl) was added to the upper compartment of the chamber and incubated with RPMI-1640 with 10% FBS in the lower compartment for 24 h at 37˚C. The filters were fixed with 4% paraformaldehyde and stained with hematoxylin. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded through the Matrigel and the filter to the lower surface were counted. Each assay was conducted in triplicate. In some experiments, anti-AMF IgG (50 μg/ml) or preimmune IgG (50 μg/ml) was incubated in the medium.

Glucose consumption, lactate production, and pH value in vitro. Parental and variant cell lines were grown to near confluence in RPMI-1640 culture medium buffered with 25 mM $NaHCO_3$ and supplemented with 10% FBS. Cultures were then rinsed with PBS and provided with a fresh culture medium. The glucose and lactate levels and pH values were subsequently assayed in the conditioned medium by a blood gas analyzer (Stat Profile M; Nova Biomedical, Waltham, MA) every 2 h.

Orthotopic tumor model

Animals and orthotopic implantation of tumor cells. Female athymic nude mice were housed under specific pathogen-free conditions and used at 6 weeks of age. Subcutaneous xenografts were established by injection of $1x10^6$ tumor cells in both flanks of 2-3 animals per cell line, and the growth was monitored regularly. Tumors were selected for orthotopic implantation at an average size of 1 cm^3, and tissue was cut into 2x2x2 mm pieces. Superficial regions of the tumors were used for implantation, and all sampled tumors were examined histologically to confirm that areas of the tumor corresponding to the sampled regions contained viable tissue. The cecum was exteriorized through a small midline laparotomy, and a piece of tumor tissue was sutured to the cecal surface with a single Maxon™ 6-0 suture, leaving the tumor tissue buried in the cecal wall. After implantation, the abdominal wall was closed in two layers. The volume (V) of the xenograft was calculated by the following formula: V = _/6(a x b x c), where a, b, and c represent the length, width, and height of the mass, respectively. The mean values and the SDs of the tumor volumes were calculated. Ten mice were inoculated with each type of cell in two separate experiments.

Histopathologic and immunohistochemical study. The tumors were fixed in 10% phosphate-buffered formalin, and 4-μm paraffin-embedded sections were prepared. Slides were stained with H&E according to standard laboratory protocols. We completed an immunohistochemical study using a Histofine SAB-PO Kit according to the manufacturer's instructions (Nichirei Co., Tokyo, Japan).

Clinical samples. Colorectal cancer tissues were obtained from 10 patients undergoing initial surgical resection without any prior therapy. Formalin-fixed, paraffin-embedded tissue sections were subjected to immunostaining using anti-AMF IgG as described above.

Statistical analysis. A Student's t-test was used in the statistical analyses. $P<0.05$ was considered significant.

Results

Constitutive overexpression of PGI in DLD-1 transfectants. After transfection, three stably transfected cell clones (DLD-PGI1, DLD-PGI2, and DLD-PGI3) of human PGI cDNA exhibiting high-level expression of AMF in DLD-1 cells were selected and established. The PGI ratios of each clone were 3 to 4 compared with the empty vector-transfected control (Fig. 1). No differences in expression of AMFR were observed among the PGI transfectants and vector-only transfected cells. We conducted tests to determine whether overexpression of PGI in DLD-1 human colon cancer cells affects their *in vitro* growth properties. No statistical significance in the growth rate among the cell variants was detected (data not shown). PGI increased the invasive ability of DLD-1 cells by 1.5- to 2-fold relative to the parental DLD-1 and DLD-zeo (Fig. 1C).

In vitro metabolism of parental and PGI-overexpressing cells. The glucose metabolism in the PGI-overexpressing and

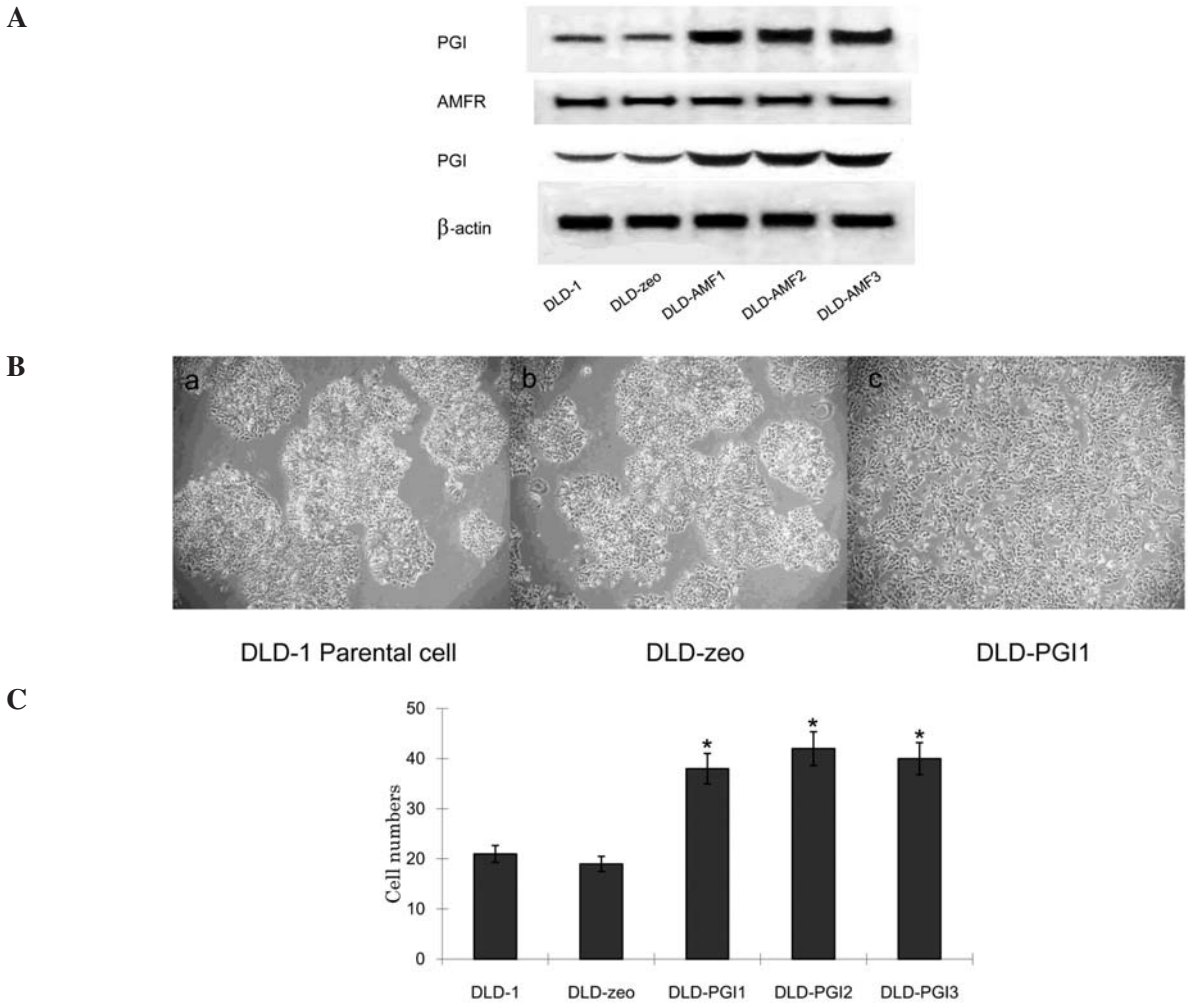

Figure 1. (A) PGI expression in colon cancer cells transfected with PGI expression vectors. The parental DLD-1 cells were stably transfected with empty vector (DLD-zeo), and AMF expression vector (DLD-PGI1, DLD-PGI2, and DLD-PGI3, three individual clones), and cell lysates from these cells were subjected to Western blot analysis with anti-PGI antibody (top row) or anti-AMFR antibody (second row). The secretion of AMF was analyzed by Western blotting of 50 μg of protein from conditioned medium (third row). The fourth row was probed with anti-ß-actin antibody as a control. (B) Morphological changes induced by PGI overexpression. Morphological alterations after PGI overexpression were observed in the transfected cell lines. Parental DLD-1 (a) and DLD-zeo (b) cells were more adherent to each other and formed compact clusters, whereas the PGI-transfected cells (c) continued to exhibit a dispersed morphology. (C) Invasive ability of PGI-transfected DLD-1 cells. A single-cell suspension (200 μl) (1x10^6 cells/ml) of cells was placed in the upper wells of individual transwell inserts containing 8-μm pore polycarbonate membranes precoated with Matrigel. Cells were allowed to invade for 24 h at 37˚C, and then they were fixed and stained with hematoxylin. Cells on the upper surface were removed with a cotton swab, and the cells that migrated to the lower side of the membrane were mounted on a microscope slide and counted under a light microscope at x200 magnification. *In vitro* invasive ability was significantly greater in PGI-transfected cells when compared with parental and empty vector-transfected DLD-1 cells at 24 h. *Significant difference when compared with the control (P<0.005). Each value represents a mean of three readings. Error bars represent 95% confidence intervals. *P<0.001.

Table I. Effect of PGI overexpression on colonic tumors in nude mice.

	Cecal tumors		Liver metastasis
Cell line	Incidence	Volume (mm^3)	Incidence
DLD-1	10/10	39.3±3.5	0/10
DLD-zeo	10/10	36.7±1.9	0/10
DLD-PGI1	10/10	93.1±13.4[a]	6/10
DLD-PGI2	10/10	92.3±16.5[a]	5/10
DLD-PGI3	10/10	90.9±19.3[a]	6/10

[a]P<0.01 versus PGI-zeo.

control vector clones was compared by measuring the glucose and lactate levels and pH values in an *in vitro* time course assay. Both glucose consumption and lactate production were significantly lower in the parental and vector-only cells than in the PGI-overexpressing cells (Fig. 2A and B). The PGI-overexpressing cells acidified the medium more strongly than did the parental and vector-only cells (Fig. 2C).

Effect of PGI expression on the in vivo growth of DLD-1 cells. To determine the metastatic potential of parental DLD-1, DLD-zeo, and DLD-PGI cells (2x10^6 cells/mouse), these cells were orthotopically implanted into the cecum of nude mice (n=10 for each variant). Orthotopically implanted control cells (parental DLD-1 and DLD-zeo) formed small tumors, and PGI-overexpressing cells produced larger tumors (Fig. 3Aa and b). The metastatic potential of the cells was

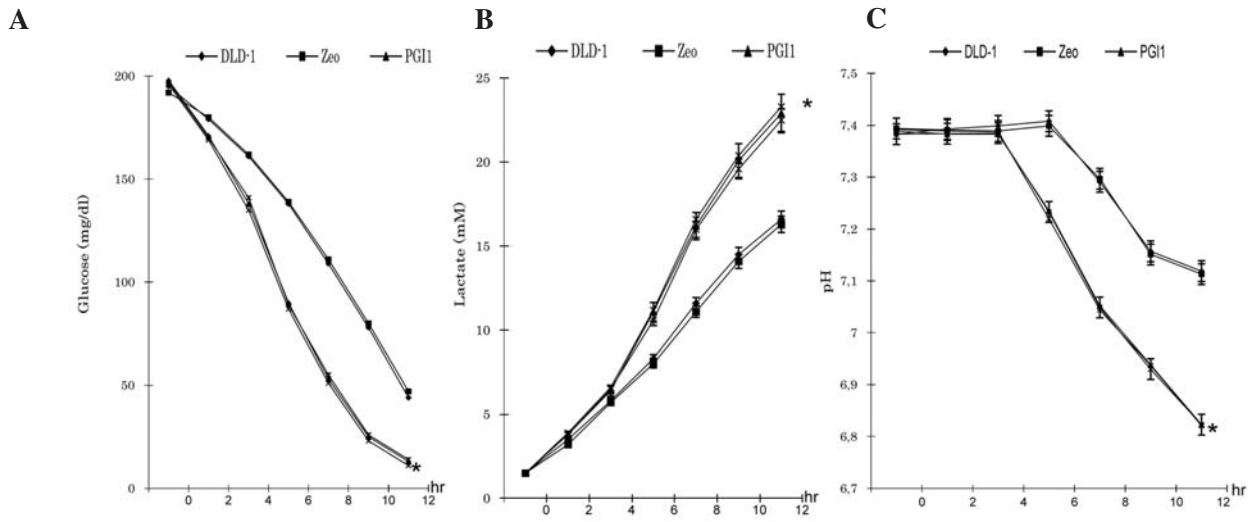

Figure 2. Glucose consumption (A), lactate production (B), and medium acidification (C) of the derived DLD-1, DLD-zeo and DLD-PGI cells *in vitro*. Cells were grown as described in Materials and methods. The mean ± SD (bars) for each time point is shown (n=3). *Significant difference when compared with the control (P<0.0001 at 12 h).

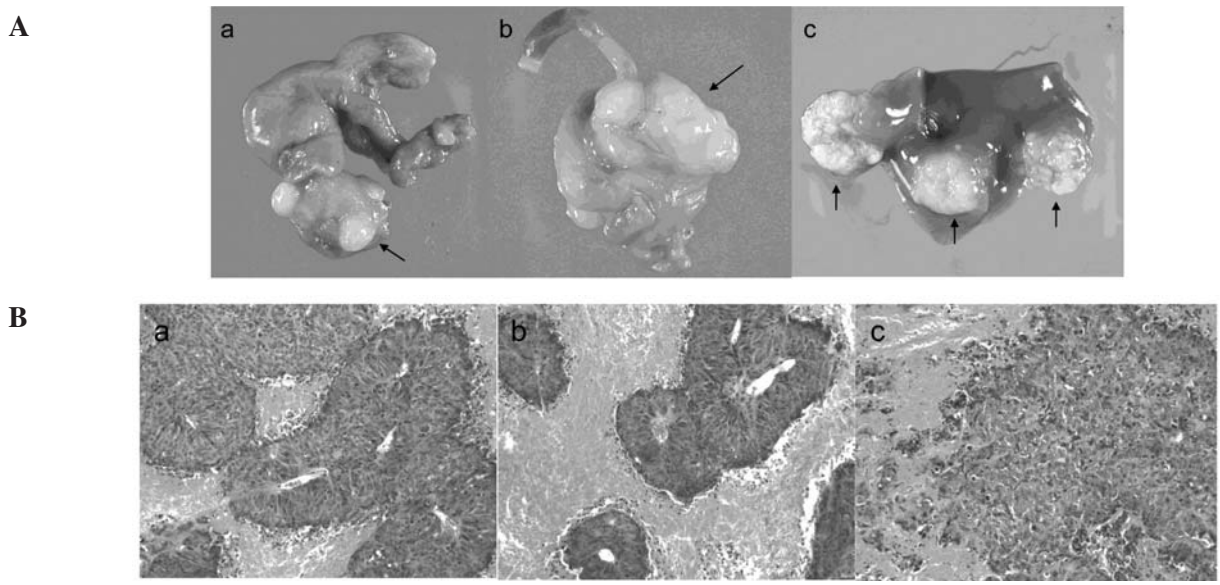

Figure 3. (A) PGI enhanced growth and metastasis *in vivo*. To evaluate the effect of PGI signaling on local and distant metastatic tumor growth, a more clinically relevant orthotopic tumor model was selected. Empty vector-transfected DLD-1 cells formed small tumors (a, arrow), whereas the PGI-transfected cells formed more aggressive tumors (b, arrow). PGI-transfected DLD-1 cells produced spontaneous liver metastases (c, arrow), whereas the parental and empty vector-transfected DLD-1 cells were nonmetastatic in a reproducible manner. (B) Histologic appearance of PGI-overexpressing tumors in nude mice. Tumors were generated by orthotopical inoculation of PGI-transfected DLD-1 cells as described in Materials and methods. Parental (a) and empty vector-transfected (b) DLD-1 tumors did not invade surrounding tissues and showed localized tumor growth, whereas PGI-transfected cells focally invaded the surrounding colonic tissue (c).

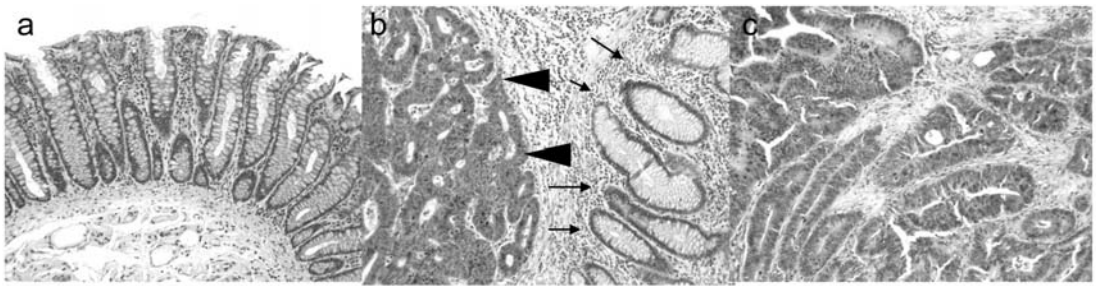

Figure 4. Expression of PGI in human colon cancers. Representative example of PGI expression of human colon cancer determined by immunohistochemistry. a, Normal colonic mucosa; b, moderately differentiated adenocarcinoma (arrow); c, moderately differentiated adenocarcinoma. Strongly positive PGI expression is present in the cytoplasm of cancer cells (b and c) but not in normal colonic epithelial cells (a).

determined 1 month after orthotopic implantation of tumor cells. DLD-PGI cells produced spontaneous liver metastases (Fig. 3Ac), whereas the parental DLD-1 and DLD-zeo cells were never metastatic. These data show that increased expression of PGI leads to the enhancement of tumor formation and metastasis of human colon cancer cells (Table I). Parental DLD-1 and DLD-zeo tumors did not invade surrounding tissues and showed localized tumor growth (Fig. 3Ba and b), whereas DLD-PGI cells focally invaded surrounding colonic tissue (Fig. 3Bc).

Overexpression of PGI in human colon cancer. Expression of the PGI protein was examined in paraffin sections from 10 colon cancer patients by immunohistochemistry. Strong cytoplasmic staining of the PGI protein was detected in 10 cancers but weakly expressed in normal colonic epithelium (Fig. 4). A representative example of PGI expression in human colon cancer was determined by immunohistochemistry. Strongly positive PGI expression was observed in the cytoplasm of cancer cells (Fig. 4b and c) but not in that of normal colonic cells (Fig. 4a).

Discussion

The recent advent of new agents for the treatment of metastatic CRC has enhanced the therapeutic strategy for this disease. Oxaliplatin in the FOLFOX and FOLFIRI regimen has been shown to be effective in achieving an improved response and time to progression (3). The monoclonal antibodies bevacizumab (targeting the vascular endothelial growth factor) and cetuximab (targeting EGFR) have also shown therapeutic efficacy in CRC (4). The effort to further improve the efficacy and tolerability of treatment for metastatic CRC has led to the discovery of new agents targeting cell-signaling molecules.

Cancer metastasis is a multistep process involving complex and highly coordinated interactions between tumor cells and a constantly changing host microenvironment (12). It has also been reported that the aberrant expression of glycolytic enzymes is regulated by hypoxia, including PGI expression (13). The adaptation of cancer cells to hypoxia is regulated by hypoxia-inducible factor-1, a key transcription factor that up-regulates the glycolytic enzyme that improves cell survival and promotes the progression of cancers that rely on aerobic glycolysis (14). Likewise, the increased expression of this glycolytic enzyme supports the survival and growth of these cells under hypoxic conditions in cancers (15). The demonstration that PGI-overexpressing DLD-1 cells exhibit enhanced glucose consumption and lactate production properties *in vitro* highlights the possible role of PGI overexpression during cancer progression and metastasis. Targeting of growth factor receptors with monoclonal antibodies for cancer treatment has become possible with the recent introduction of humanized antibodies.

The present study highlights the role of PGI expression in a key event which controls tumor progression and metastasis. The results herein call for the development of specific anti-PGI antibodies or a PGI inhibitor specifically targeting metastatic CRC. Such inhibitors are being actively investigated at the molecular level and may hold promise as effective anticancer treatments. In addition, research is required to determine whether PGI can be a therapeutic target candidate.

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