Growth promotion of HepG2 hepatoma cells by antisense-mediated knockdown of glypican-3 is independent of insulin-like growth factor 2 signaling

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Abbreviations: GPC3, glypican-3; IGF2, insulin-like growth factor 2

Abstract

Glypican-3 (GPC3) encodes a cell-surface heparan-sulfate proteoglycan and its expression is frequently silenced in ovarian cancer, mesotheliomas, and breast cancer cell lines and ectopic expression of GPC3 inhibited the growth of these cells, suggesting that GPC3 plays a negative role in cell proliferation. In contrast, up-regulation of GPC3 is often observed in hepatoma, neuroblastoma, and Wilms' tumor. Whether GPC3 plays the same growth inhibitory role in these tumors remains to be studied. Here we report that antisense-mediated knockdown of GPC3 in the HepG2 hepatoma cells significantly promotes the growth of hepatoma cells. In addition, we show that this growth promotion is independent of insulin-like growth factor 2 (IGF2) signaling. Our data suggest that GPC3 plays a growth-suppressing role in hepatoma and provide cell biological evidence inconsistent with the hypothesis that GPC3 acts as a growth suppressor by downregulating IGF2.

Keywords: glypican-3; growth suppression; hepatoma; insulin-like growth factor 2

Introduction

GPC3 is a member of glypican family of heparan sulfate proteoglycans, which are linked to the cell surface

through a glycosylphosphatidylinositol (GPI) anchor. GPC3 mutation and loss-of-function is known to cause type 1 Simpson-Golabi-Behmel syndrome (SGBS1), an X-linked condition characterized by pre- and post-natal overgrowth (Pilia *et al.*, 1996). Indeed, GPC3 knock out mice exhibited several phenotypic features of SGBS1 including somatic overgrowth, renal dysplasia, accessory spleens, polydactyly, and placentalomegaly (Cano-Gauci *et al.*, 1999; Paine-Saunders *et al.*, 2000; Chiao *et al.*, 2002). These findings together with cell line-specific promotion of apoptosis by rat GPC3 (Gonzalez *et al.*, 1998) suggest that GPC3 plays a negative role in cell proliferation and an apoptosis inducing role in specific tissues. Consistent with this idea, GPC3 is frequently silenced in ovarian cancer, mesotheliomas, and breast cancer cell lines and ectopic expression of GPC3 inhibited the growth of these cells (Lin *et al.*, 1999; Murthy *et al.*, 2000; Xiang *et al.*, 2001).

Beckwith-Wiedemann syndrome (BWS), another overgrowth disorder, shares significant similarities with SGBS1. Since BWS is thought to be associated with over-expression of insulin-like growth factor 2 (IGF2), it has been proposed that GPC3 may bind and sequester or downregulate IGF2 (Hughes-Benzie *et al.*, 1996; Pilia *et al.*, 1996). This hypothesis was further strengthened since mice overexpressing IGF2 showed features of SGBS1 (Eggenschwiler *et al.*, 1997). Also, some biochemical data supported this hypothesis (Pilia *et al.*, 1996; Xu *et al.*, 1998).

In contrast to the frequent silencing in ovarian cancer, mesotheliomas, and breast cancer, GPC3 is frequently upregulated in hepatocellular carcinoma (Hsu *et al.*, 1997; Zhu *et al.*, 2001; Sung *et al.*, 2003), neuroblastoma and Wilms' tumor (Saikali and Sinnott, 2000), and hepatoblastoma (Toretzky *et al.*, 2001). The role of upregulated GPC3 in these tumors is not known and remains to be studied. Here we examined effects of antisense mediated knockdown of GPC3 on the growth of Hep G2 hepatoma cells to find out role of GPC3 in hepatoma. In addition, we examined whether blocking endogenous GPC3 changes growth-stimulatory effect of IGF2 in Hep G2 cells to verify the hypothesis that GPC3 acts as a growth suppressor by downregulating IGF2.

Materials and Methods

Cell lines

The HepG2 (ATCC HB 8065) cells and SNU475 cells (Park *et al.*, 1995) were purchased from Korea Cell Line Bank (Seoul, Korea). The HepG2 cells were grown in DMEM and SNU475 cells were cultured in RPMI 1640. Medium was supplemented with 10% FBS.

RNA preparation and RT-PCR

Total RNA was isolated from the cells using TRIzol reagent (Gibco-BRL, Grand Island, NY). cDNA was synthesized from 2 µg total RNA using the cDNA synthesis kit containing the superscript II reverse transcriptase and random hexamers (Gibco-BRL). Small aliquots of the cDNA product were amplified with gene-specific primer pairs. The primers for GPC3 amplification (250 bp) are as follows: GPC3-F 5'-GAT-ACAGCCAAAAGGCAG-3', GPC3-R 5'-ATCATTCCAT-CACCAGAG-3'. β-actin was amplified as an internal control. The primers for β-actin amplification (381 bp) are β-actin-F 5'-ACCATGGATGATGATATCGC-3' and β-actin-R 5'-ACATGGCTGGGGTGTGAAG-3'. PCR amplification was performed after an initial denaturation of 5 min at 94°C, followed by 32 cycles of 30 s at 94°C, 45 s at 58°C and 1 min at 72°C, and final elongation at 72°C for 10 min.

GPC3-antisense expression vector construction

Full-length GPC3 cDNA was prepared by RT-PCR and subcloned into *Bam*HI and *Eco*RI sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA) in antisense orientation generating pGPC3-AS vector. The orientation and accuracy of sequences were confirmed by sequencing. Primer set for PCR was GPC3F (5'-CGGGATCCTCA-GTGCACCAGGAAGAAGAAGCA-3') and GPC3R (5'-CGGAATTCAGCAGGATGGCCGGGACCGTGCGCA-3'). The *Bam*HI (GGATCC) and *Eco*RI (GAATTC) sites are underlined.

Stable transfection

HepG2 cells were grown to 50-80% confluency and transfected with GPC3-antisense expression vector (pGPC3-AS) by using Lipofectamine reagent (Gibco-BRL) as described (Cho *et al.*, 2002). After 2 days, transfected cells were split 1:10 into selection medium (DMEM with 10% FBS with 400 µg/ml G418). After 3-4 weeks, clones were harvested and further expanded in selection medium. HepG2 cells were also transfected with an empty vector (pcDNA3.1) as a control.

Immunoblot analysis

Polyclonal rabbit antibody against human GPC3 synthetic peptide (AA 71-82, N-RKMEEKYQLTAR-C) was generated from GeneMed Synthesis Inc. (San Francisco, CA) and affinity purified through Protein A affinity column (Pierce, Rockford, IL). For pre-absorption, 10-fold molar excess of peptide antigen was mixed with antibody at 4°C for 16 h. Total cell lysates were prepared as described (Sung *et al.*, 2002) and separated (20 µg/lane) on 10% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked with 6% milk in PBS for 1 h, and then probed with GPC3 antibody (1 µg/ml). Horseradish peroxidase conjugated donkey anti-rabbit Ig (Amersham, Buckinghamshire, UK) was used as a secondary antibody at a 1:5,000 dilution. Bands were visualized using ECL Plus (Amersham). Membranes were stripped and reprobed with mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA) to control the integrity and quantity of the protein samples.

Growth assay

MTT assay was performed as described (Shin *et al.*, 2002). Cells were plated at a density of 5,000 cells/well in 96-well plate and incubated until indicated time. MTT (70 µg/well) was added for 4 h and the produced formazan was solubilized by DMSO. Optical density was measured at 570 nm with a microplate reader. For manual counting, cells were plated at a density of 50,000 cells/well in 6-well plate and incubated for 4 days. For IGF2 response assay, cells were plated overnight at a density of 5,000 cells/well in 96-well plates, and subsequently incubated in serum-free media in the presence of various concentrations of recombinant human IGF2 (R&D Systems, Minneapolis, MN) for 48h before adding MTT.

Statistical analysis

The t-test was used to determine statistical difference. Probability (*P*) values of less than 0.05 were considered to be statistically significant.

Results

Glypican-3 expression in HepG2 hepatoma cells

HepG2 hepatoma cell line is well known to express high level of GPC3 (Mast *et al.*, 1997; Gonzalez *et al.*, 1998; Saikali and Sinnett, 2000). Consistent with this, we detected GPC3 transcript in HepG2 cells (Figure 1A). Immunoblot analysis with anti-GPC3 antibody showed a major band with approximate molecular weight of 66 kDa, which is estimated GPC3 core protein size, in HepG2 hepatoma cells (Figure 1B).

Figure 1. RT-PCR (A) and Immunoblot (B) detection of glypican-3 expression in HepG2 hepatoma cells. The SNU475 cell line was used as a negative control. (C) Antisense-mediated knockdown of GPC3 expression in HepG2 cells. Immunoblot using GPC3 antibody with (lane 1) or without (lane 2) addition of the peptide antigen to confirm the specificity of the antibody. Immunoblot with empty vector-transfected control pool cells (lane 3), and GPC3-antisense transfected AS pool cells (lane 4). Both core (arrow) and glycanated (star) GPC3 were significantly reduced in GPC3-antisense transfected AS pool cells. (D) Direct counting showed growth promotion due to antisense-mediated knockdown of GPC3. Cells were seeded at a density of 50,000 cells/well in 6-well plate and manually counted over 4 days of culture. Values represent the means $\pm$ standard error of triplicate wells. (E) MTT assay also showed increased cell proliferation in AS pool cells. Cells were seeded at a density of 5,000 cells in 96-well plates and growth of parental (black bar), control pool (white bar), and antisense pool (gray bar) was measured over 5 days of culture. Values represent the mean $\pm$ standard error of eight determinations per experiment from two independent experiments (*, $P < 0.01$ as compared with parental HepG2 and control pool).

Antisense-mediated knockdown of glypican-3 in HepG2 hepatoma cells

To investigate the role of GPC3 in hepatoma, we decided to block endogenous GPC3 in HepG2 cells by antisense-mediated knockdown. Accordingly, we constructed GPC3-antisense expression vector (pGPC3-AS) and transfected HepG2 cells and selected in the presence of neomycin. Surviving clones were pooled and expanded and named as 'AS pool'. HepG2 cells were also transfected with an empty vector (pcDNA3.1) as a control and named as 'control pool'. Densitometry of immunoblot showed that GPC3 protein level in AS pool was 10% and 15% of parental Hep G2 and empty vector transfected control pool, respectively (Figure 1C, compare lane 4 with lanes 2 and 3). In addition, we noticed that glycosylated GPC3 is no longer detected in AS pool, demonstrating successful blocking of endogenous GPC3 in HepG2 cells

by antisense-mediated knockdown (Figure 1C).

Increased cell proliferation by antisense-mediated knockdown of GPC3

We next investigated whether blocking endogenous GPC3 changes HepG2 cell growth. Manual counting (Figure 1D) and MTT assay (Figure 1E) showed significant growth promotion in the AS pool compared to parental HepG2 and control pool. These data show that GPC3 protein level affects HepG2 cell growth and demonstrate growth inhibitory role of GPC3 in HepG2 cells.

Increased cell proliferation by blocking endogenous GPC3 is independent of IGF2 signaling

Since it has been proposed that GPC3 may bind and

Figure 2. Increased cell proliferation by blocking endogenous GPC3 is independent of IGF2 signaling. (A) Dose-dependent growth-stimulatory effect of IGF2 on parental HepG2 cells. Cells were seeded at a density of 5,000 cells/well in 96-well plates and incubated for 48 h with the indicated concentrations of IGF2. Data are expressed as percent change from unstimulated controls and are the mean $\pm$ standard error of six determinations per experiment from two independent experiments. (B) IGF2 response assay. Parental, control pool, and antisense pool cells were incubated in the absence or presence of indicated concentrations of IGF2. Data are expressed as percent change from unstimulated controls and are the mean $\pm$ standard error of ten determinations per experiment from three independent experiments.

sequester or downregulate IGF2 (Hughes-Benzie *et al.*, 1996; Pilia *et al.*, 1996), we next investigated whether the increased cell proliferation by antisense-mediated knockdown of GPC3 is due, at least in part, to enhanced IGF2 signaling. We assumed that if GPC3 downregulates IGF2, reduced expression of GPC3 would increase growth stimulatory action of IGF2. IGF2 stimulated the proliferation of the parental HepG2 cells in a dose-dependent manner (Figure 2A). With various concentrations of IGF2, we observed that growth-stimulatory action of IGF2 was similar in parental HepG2 cells, control pool, and AS pool as shown in Figure 2B. These data show that growth-stimulatory effect of IGF2 does not depend on GPC3 protein level and demonstrate that increased cell proliferation by antisense-mediated knockdown of GPC3 is not due to enhanced IGF2 signaling.

Discussion

In this study, we showed that antisense-mediated knockdown of GPC3 significantly promotes the growth of HepG2 hepatoma cells, suggesting that GPC3 plays a growth-suppressing role in hepatoma. Since GPC3 is frequently upregulated in hepatoma (Hsu *et al.*, 1997; Toretsky *et al.*, 2001; Zhu *et al.*, 2001), the growth inhibitory role of GPC3 in HepG2 cells was unexpected. However, growth-suppressing activity of GPC3 is consistent with the growth inhibition by ectopic expression of GPC3 in ovarian cancer, mesothelioma, and breast cancer cell lines (Lin *et al.*,

1999; Murthy *et al.*, 2000; Xiang *et al.*, 2001). In addition, we observed that ectopic expression of GPC3 resulted in about 50% inhibition of colony-forming efficiency in SNU475 cells (unpublished observation). It would be worth to check whether GPC3 plays similar role in cell lines derived from Wilms' tumor and neuroblastoma, where GPC3 is also frequently over-expressed.

Since GPC3 is linked to the cell surface and known to bind/modulate several growth factors through heparan sulfate chains (Song *et al.*, 1997; Paine-Saunders *et al.*, 2000; Grisaru *et al.*, 2001), mechanism of growth suppression by GPC3 in HepG2 cells can be explained by two ways. One is that GPC3 can be a co-receptor helping the binding of growth-inhibitory factor(s). Alternatively, GPC3 may sequester or downregulate growthpromoting factor(s). Actually, it has been hypothesized that GPC3 may bind and sequester or downregulate IGF2 (Hughes-Benzie *et al.*, 1996; Pilia *et al.*, 1996) and this hypothesis was supported by some data (Pilia *et al.*, 1996; Eggen-schwiler *et al.*, 1997; Xu *et al.*, 1998). However, our data in this study show that growth-stimulatory effect of IGF2 does not depend on GPC3 protein level in Hep G2 cells. Our data suggest that IGF2 signaling is not modulated by GPC3 and that growth promotion induced by blocking endogenous GPC3 is independent of IGF2 signaling. The recent paper by Chiao *et al.*, (2002) providing genetic evidence that over-growth of a mouse model of SGBS1 is independent of IGF signaling support our data. Moreover, consistent with our data, Midorikawa *et al.* (2003) recently

showed that enhanced expression of GPC3 was not able to alter cell proliferation induced by IGF2.

In conclusion, we demonstrate here that GPC3 inhibits the proliferation of hepatoma cells *in vitro*, and suggest that GPC3 may not be implicated in the development of hepatoma at least through a signaling pathway that involves IGF2.

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