SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 26: 743-749, 2011

Down-regulation of β-*N*-acetyl-D-glucosaminidase increases Akt1 activity in thyroid anaplastic cancer cells

ANNA KRZEŚLAK, PAWEŁ JÓŹWIAK and ANNA LIPIŃSKA

Department of Cytobiochemistry, University of Lodz, 90-236 Lodz, Poland

Received April 14, 2011; Accepted May 9, 2011

DOI: 10.3892/or.2011.1333

Abstract. *O*-GlcNAcylation is a common and dynamic modification of intracellular proteins in which β-*N*-acetylglucosamine moieties are attached to hydroxyl groups of serine or threonine residues (*O*-GlcNAc). Accumulating evidence suggests the critical role of protein *O*-GlcNAcylation in signal transduction, transcriptional control, cell cycle regulation and protein degradation. However, the exact role of *O*-GlcNAc modification in tumor pathogenesis or progression remains to be established. In the present study, we investigated the effect of increased *O*-GlcNAcylation of cellular proteins on IGF-1 signaling in 8305C thyroid anaplastic cancer cells. The global *O*-GlcNAc level in the 8305C cells was increased by down-regulation of β-*N*-acetyl-D-glucosaminidase (*O*-GlcNAcase) activity, an enzyme which removes *O*-GlcNAc moieties. We demonstrated here that IGF-1 stimulates Akt1 activity in 8305C cells, and down-regulation of *O*-GlcNAcase activity by the chemical inhibitor PUGNAc or RNA interference method enhances this effect. Increased Akt1 activation increased cell proliferation. In cells with down-regulation of *O*-GlcNAcase activity, kinase GSK3β phosphorylation and cyclin D_1 levels were higher than those in control cells. Our findings suggest that increased proliferation of 8305C cells treated with PUGNAc or RNAi against *O*-GlcNAcase at least partially depends on the IGF-1-Akt1-GSK3β-cyclin D_1 pathway.

Introduction

The insulin-like growth factor 1 (IGF-1) signaling pathway plays important roles in regulating cellular proliferation and apoptosis (1,2). IGF-1 activates the phosphatidylinositol 3-kinase/Akt and extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways. Upon the binding of IGF-1 to the IGF-1 receptor, the receptor becomes autophosphorylated on several tyrosine residues. These phosphotyrosines serve as binding sites for adaptor proteins, including insulin receptor substrate (IRS) and Shc, which are then phosphorylated by the activated receptor. Phosphorylated IRS and Shc in turn recruit Grb2/SOS, which leads to activation of the MAPK pathway and phosphatidylinositol 3-kinase, resulting in phosphatidylinositol 3,4,5-triphosphate production and Akt activation (2).

Aberrations in the generation or action of IGF-1 have been suggested to play an important role in several pathological conditions, including metabolic disorders, neurodegenerative diseases and different types of cancers (1). Overexpression and increased tyrosine kinase activity of the IGF-1 receptor (IGF-1R) have also been noted in many human thyroid cancers (3,4).

Protein post-translational modifications represent an important mechanism for the regulation of cellular physiology and function. One of the most common modifications of intracellular proteins is *O*-GlcNAcylation in which β-*N*-acetylglucosamine moieties are attached to hydroxyl side chains of serine or threonine residues of proteins (5-7). Unlike most carbohydrate modifications, *O*-GlcNAcylation is very dynamic and inducible and as such it is more similar to phosphorylation (5). Accumulating evidence suggests the critical role of *O*-GlcNAcylation in signal transduction, transcriptional control, cell cycle regulation, protein degradation and stress responses (6,7). The role of *O*-GlcNAcylation in signal transduction is in part related to its competitive interaction with *O*-phosphorylation. Some of the known *O*-GlcNAc sites are the same as or adjacent to phosphorylation (5).

Reversible *O*-GlcNAcylation is catalyzed by the nucleocytoplasmic enzymes, *O*-β-*N*-acetylglucosaminyltransferase (OGT) and β-*N*-acetylglucosaminidase (*O*-GlcNAcase), which adds or removes *O*-GlcNAc moieties, respectively (7). Down-regulation of *O*-GlcNAcase activity is a commonly used approach for elevating a global *O*-GlcNAc level in cells during studies concerning the biological role of this modification.

Abnormally regulated *O*-GlcNAcylation has been implicated in diseases such as diabetes, Alzheimer's disease and cancer (7,8). However, the role of *O*-GlcNAcylation in tumorigenesis and the progression of cancer is still under-investigated.

In the present study, we investigated whether increased *O*-GlcNAcylation of cellular proteins affects IGF-1 signaling in 8305C thyroid anaplastic cancer cells. Our results demonstrated,

Correspondence to: Dr Anna Krześlak, Department of Cytobiochemistry, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland
E-mail: krzeslaka@interia.pl

Key words: *O*-GlcNAcylation, thyroid cancer, Akt1, IGF-1, proliferation

for the first time, that down-regulation of *O*-GlcNAcase enhances IGF-1-stimulated Akt1 activation which promotes proliferation of thyroid cancer cells.

Materials and methods

Reagents and antibodies. All chemicals were obtained from Sigma (St. Louis, MO) except as noted. Cell culture reagents and materials were purchased from Invitrogen (Carlsbad, CA) and Corning, Inc. (Corning, NY). General anti-*O*-GlcNAc mouse IgM monoclonal CTD 110.6 antibodies were purchased from Covance (Princeton, NJ). Rabbit polyclonal antibodies against phospho-GSK3β (Ser9) and phospho-Akt (Ser473) were from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-Akt1, anti-cyclin D_1, anti-β-actin and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments. 8305C thyroid anaplastic cancer cells (ECACC) were grown in Advanced MEM medium suplemented with 2 mM glutamine and 5% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells were treated with 100 μM of the *O*-GlcNAcase inhibitor PUGNAc [O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate] for 24 or 48 h.

Two small interfering RNA duplexes that target the coding region of human *O*-GlcNAcase mRNA sequences (GenBank accession no. AF036144) were used to suppress the enzyme level in the 8305C cells (siOGA1: sense, 5'-GCCACCUGGUAC AAAUCAAdTdT; antisense, 5'-UUGAUUUGUACCAGGUG GCdTdT; siOGA2: sense, 5'-CCGUAGUAACAACAGUUU AdTdT; antisense, 5'-UAAACUGUUGUUACUACGGdTdT). In the negative controls, scrambled non-silencing siRNA was used (sense, 5'-UUCUCCGAACGUGUCACGUdTdT; antisense,5'-ACGUGACACGUUCGGAGAAdTdT). Transfections were carried out using Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's instructions. Cells were treated with 30 nM of siRNAs, and the effect of interference was assessed after 24 or 48 h. Cells were cultered in serum-free medium for 24 h prior to treatments with IGF-1 for 20 min.

Real-time RT-PCR. RNA was isolated from the 8305C cells using the Total RNA Isolation kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. First Strand cDNAs were obtained by reverse transcription of 1 μg of total RNA using RevertAid™ First Strand cDNA synthesis kit (Fermentas International, Lithuania) following the manufacturer's protocol. Real-time amplification of the cDNA was performed using TaqMan® Gene Expression Assay (Applied Biosystems) according to the manufacturer's instructions. The fluorogenic, FAM-labeled probes and the sequence-specific primers for *MGEA5* (gene coding *O*-GlcNAcase) and the internal control *GAPDH* were obtained as inventoried assays (Hs 00201970 and Hs99999905, respectively). Fold differences in *O*-GlcNAcase expression, normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels were calculated using the formula $2^{\Delta\Delta Ct}$. Relative amounts of mRNA in siRNA-treated cells are indicated as the percentage of the amount of mRNA in the untreated cells.

O-GlcNAcase activity assay. *O*-GlcNAcase activity assay was performed at a pH of 6.45 using *p*-NP-GlcNAc (4-nitrophenyl *N*-acetyl-D-glucosaminide) as a substrate. The supernatant samples of the cell lysates were mixed with 200 μM β-*N*-acetyl-galactosamine in a proportion 1:1 and incubated for 15 min at 37˚C. Then 100 μl of 2 mM substrate in MES [2-(*N*-morpholino) ethanesulfonic acid] buffer was added, and the samples were incubated at 37˚C for 3 h. The reaction was stopped with the addition of sodium carbonate. The color was measured spectrophotometrically at 400 nm with the blanks routinely subtracted.

Western blotting. Proteins of the cell lysates were resolved by 8% SDS-PAGE and electroblotted onto Immobilon-P transfer membranes. The blots were incubated with primary antibodies overnight at 4˚C. After washing three times with TBS (Tris-buffered saline) blots were incubated for 1 h with goat anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase. Proteins were visualized on X-ray film by an enhanced chemiluminescence method. For loading control, blots were reprobed with anti-β-actin antibody following a stripping protocol.

Cell proliferation assay. Cell viability and proliferation were assessed using the Quick Cell Proliferation Assay Kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's instructions. The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Cells were plated onto 96-well plates at 4000 cells per well and cultivated for 24 h in standard conditions. Then the medium was replaced with a new medium with or without FBS, and cells were treated with PUGNAc or RNAi. After 72 h of incubation, 10 μl of WST-1 reagent per well was added, and after a 1-h incubation the absorbance of the samples was measured at 450 nm using a microplate reader with a blank (medium alone) as the background control.

Statistical analysis. Where appropriate, experimental data are expressed as the mean ± SD. For comparison of groups, the Student's t-test was used. A level of $p<0.05$ was considered as statistically significant.

Results

Down-regulation of O-GlcNAcase activity. To increase the *O*-GlcNAcylation of proteins, 8305C cells were treated with PUGNAc which is a GlcNAc analogue that potently inhibits *O*-GlcNAcase in cells. PUGNAc, by preventing the cycling of *O*-GlcNAc on proteins, leads to globally elevated levels of this modification. 8305C cells were treated with 100 μM PUGNAc for 24 or 48 h. As expected, PUGNAc effectively increased the *O*-GlcNAc level in treated cells. However, PUGNAc is not a selective inhibitor of *O*-GlcNAcase and also inhibits the lysosomal β-hexosaminidases, enzymes that cleave both GlcNAc and GalNAc from a range of glycoconjugates (9). Thus, we used the alternative methodological approach to down-regulate *O*-GlcNAcase activity, i.e., the RNA interference method. To down-regulate the expression of *O*-GlcNAcase, two small interfering RNA duplexes (siOGA1 and siOGA2) were used. In preliminary experiments, the cells were treated with different concentrations of siRNAs for 24, 48 or 72 h (data not shown).

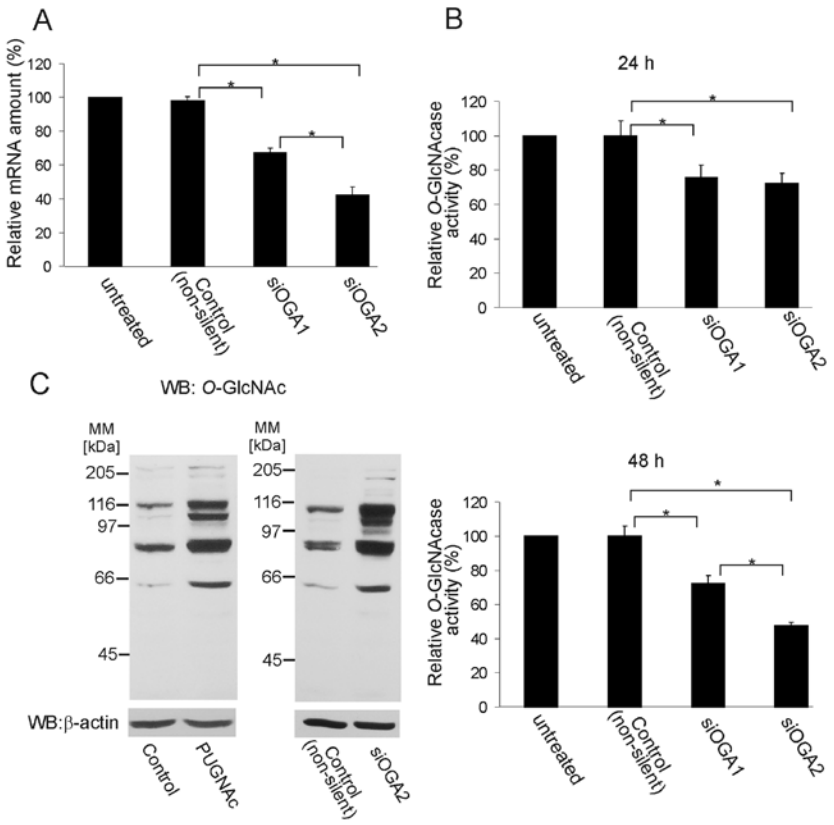

Figure 1. RNAi-mediated down-regulation of *O*-GlcNAcase. 8305C cells were treated with two different small interfering RNA duplexes (siOGA1 and siOGA2) or control scrambled siRNA. (A) Relative amount of *O*-GlcNAcase mRNA 24 h after cells were transfected with 30 nM siRNAs as assessed by real-time RT-PCR. (B) Results of the *O*-GlcNAcase activity assay 24 and 48 h after transfection. Error bars represent the mean ± SD of 2-4 independent experiments, $^{*}p<0.01$. (C) Western blot analysis of changes in the *O*-GlcNAc level of cellular proteins in lysates of 8305C cells treated with 100 μM PUGNAc or 30 nM siOGA2 for 48 h. Western blot analysis for *O*-GlcNAc was performed with CTD 110.6 antibodies. For loading control, immunodetection of β-actin was performed. Bars indicate molecular mass (MM) of the protein standards.

The concentration of 30 nM siRNA and a 48-h treatment were found to be optimal conditions. For transfection, Lipofectamine RNAiMAX reagent was used. The control cells were transfected with a non-silent scrambled RNA duplex. The effect of RNAi was assessed by real-time RT-PCR and *O*-GlcNAcase activity assay (Fig. 1A and B). Both siRNAs caused a decrease in the mRNA level and activity of *O*-GlcNAcase as well as an increase in the *O*-GlcNAc level. However, siOGA2 was more effective in reduction of the *O*-GlcNAcase level, thus it was chosen for further experiments. Since anti-*O*-GlcNAcase antibodies are not available, the *O*-GlcNAcase protein level was not analyzed. Changes in *O*-GlcNAc level were detected by Western blot analysis with specific *O*-GlcNAc CTD 110.6 antibodies. Although RNAi did not completely abolish *O*-GlcNAcase activity but reduced it only by 50-60%, the increase in *O*-GlcNAc level was similar to that caused by 100 μM PUGNAc treatment for 24 h (Fig. 1C).

Down-regulation of O-GlcNAcase activity increases Akt1 phosphorylation. After 24 h of serum starvation, 8305C cells treated with 100 μM PUGNAc or 30 nM siOGA2 were stimulated for 20 min with 100 ng/ml IGF-1. Cell lysates were resolved by SDS-PAGE, and phospho-Akt (Ser473) or Akt1 were detected with specific antibodies (Fig. 2). An increase in Ser473 phosphorylation was noted both in the cells treated with PUGNAc and siOGA2 in comparison to the control cells (untreated or treated with non-silent duplex). The reduction in *O*-GlcNAcase activity affected both basal and IGF-1-induced Akt1 phosphorylation. However, the increase in Akt1 phosphorylation was greater in cells stimulated with IGF-1.

Down-regulation of O-GlcNAcase activity affects cell viability and proliferation. To determine whether the changes in *O*-GlcNAcylation level affect cell viability and proliferation, thyroid anaplastic cancer cells were treated with PUGNAc or siRNA and grown in medium with or without serum for 72 h. Then cell proliferation assay was performed. When the 8305C cells growing in medium containing FBS were treated either with PUGNAc or siOGA2, viability/proliferation was increased by ~12 and ~20%, respectively (Fig. 3A and B). Cells growing in medium without FBS showed decreased proliferation by 35-40%. However, cells treated with PUGNAc or siRNA demonstrated higher proliferation relative to the untreated or scrambled siRNA-treated controls by 6 and 10%, respectively.

Since IGF-1 is known as one of the key factors involved in cell survival and proliferation, we investigated whether increased IGF-1-stimulated Akt1 activity in cells with down-regulated *O*-GlcNAcase activity affects cell proliferation. Untreated control cells and cells treated with PUGNAc or

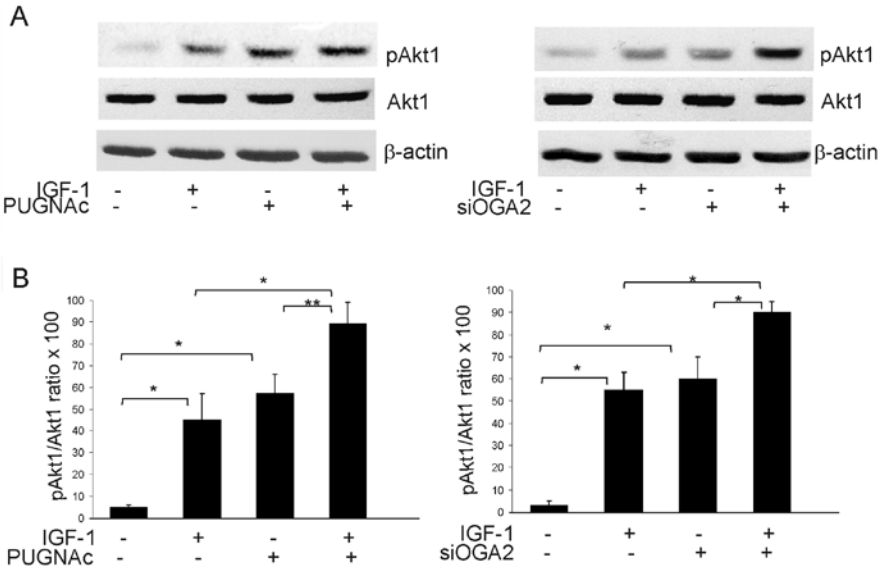

Figure 2. Down-regulation of *O*-GlcNAcase activity increases both basal and IGF-1-stimulated Akt1 phosphorylation. 8305C cells were treated with 100 μM PUGNAc or 30 nM siOGA2 and after 24 h of serum starvation, cells were stimulated for 20 min with 100 ng/ml IGF-1. (A) Cell lysates were prepared and used for Western blots for pAkt1 (Ser473), Akt1 and β-actin (loading control). (B) Intensity of protein bands was quantified by densitometry, and the ratio of phospho-Akt1 (pAkt1) to total Akt1 was estimated. Error bars represent the mean ± SD of three independent experiments; *p<0.01, **p<0.02.

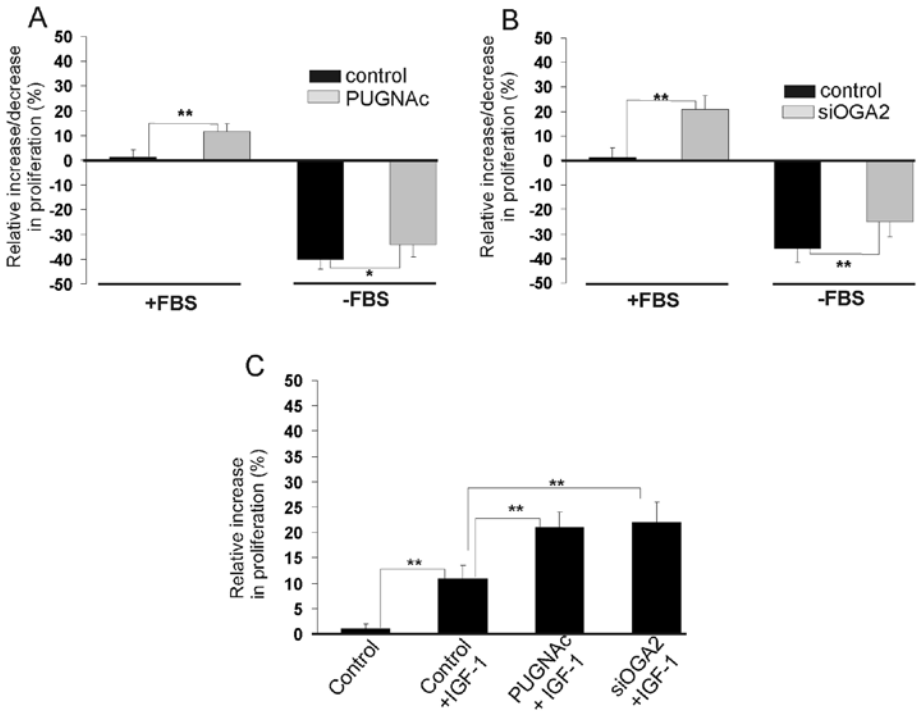

Figure 3. Down-regulation of *O*-GlcNAcase activity increases cell viability and proliferation. Control cells and cells treated with PUGNAc (A) or siRNA (B) were grown in medium with or without FBS for 72 h. A cell proliferation assay was performed, and the absorbance was measured at 450 nm. The mean optical density measured from the control (untreated or treated with non-silent siRNA) cells grown in medium with FBS was considered to be 100%. The histograms show an increase and decrease in proliferation relative to the control cells. A histogram plot represents the mean values from five repeats ± SD. (C) Serum-starved control and PUGNAc- or siRNA-treated cells were stimulated with IGF-1 for 48 h. Cells treated with IGF-1 demonstrated increased cell proliferation relative to the untreated controls. The histogram plot represents the means ± SD of three independent experiments; *p<0.05, **p<0.01.

siRNA were serum starved for 24 h and then stimulated with IGF-1 for 48 h. IGF-1 stimulation caused cell proliferation to increase by ~11% (Fig. 3C). In cells with down-regulation of *O*-GlcNAcase activity, the increase in proliferation was greater (20-22%). These results confirm the hypothesis that IGF-1 is one of the factors involved in 8305C cell proliferation.

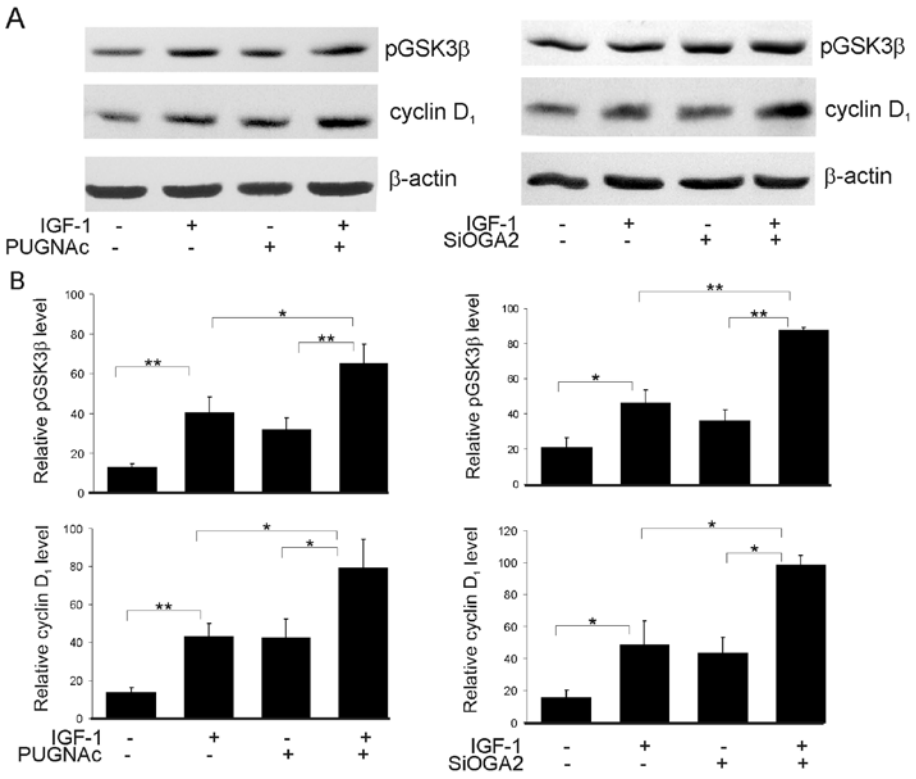

Figure 4. Effect of PUGNAc or RNAi treatment on pGSK3β (Ser9) and cyclin D_1 level in 8305C cells. Serum-starved control and PUGNAc- or siRNA-treated cells were stimulated with IGF-1 for 24 h. (A) Lysate proteins were subjected to Western blot analysis of pGSK3β and cyclin D_1 expression. (B) Quantification of pGSK3β and cyclin D_1 level by densitometry. Protein levels were normalized to β-actin. Error bars represent the mean ± SD of three experiments; $^{*}p<0.05$, $^{**}p<0.02$.

Down-regulation of O-GlcNAcase and IGF-1 stimulation affect pGSK3β and cyclin D_1 levels. It has been shown that in FRTL rat thyroid cells IGF-1 stimulation causes cyclin D_1 expression (10). We tested the possibility that an increased *O*-GlcNAc level affects the cyclin D_1 level, which is a substrate of GSK3β. Cells were serum starved for 24 h and stimulated with IGF-1 for 24 h. Cell lysates were resolved by SDS-PAGE, and pGSK3β and cyclin D_1 were analyzed by Western blotting. In the PUGNAc- and siRNA-treated cells, the pGSK3β (Ser9) level was found to be higher than that in the control cells. In the cells with down-regulated *O*-GlcNAcase activity treated with IGF-1 the level of cyclin D_1 was significant higher then that in the control cells (Fig. 4).

Discussion

Insulin-like growth factors (IGFs) are essential for cell proliferation, differentiation and survival as well as maintenance of cell functions in many cell types (11). One of the key molecules involved in the signal transduction pathways of IGF-1 is the proto-oncogene Akt, a serine and threonine kinase.

The purpose of this study was to examine the effect of an increased *O*-GlcNAc level in 8305C anaplastic thyroid cancer cells on IGF-1-induced Akt activation. It has been shown that insulin and IGF-1 signaling pathways in different cell types can be affected by changes in the cellular *O*-GlcNAc level (12-14). Increased *O*-GlcNAcylation observed under chronic hyperglycemic conditions, has been implicated in the pathogenesis of diabetes (15). The role of *O*-GlcNAc in tumor pathogenesis and progression remains to be established.

The *O*-GlcNAc level was increased in cells by down-regulation of *O*-GlcNAcase activity. Two methodological approaches were used. First, cells were treated with PUGNAc, a non-metabolizable analogue of glucosamine and a potent inhibitor of *O*-GlcNAcase (16). Prolonged cellular treatment with PUGNAc is known to elevate *O*-GlcNAc modification of numerous proteins and has been a useful tool for the study of cellular responses affected by *O*-GlcNAc modification. However, PUGNAc is not a specific inhibitor of *O*-GlcNAcase and it inhibits other lysosomal hydrolases. Few studies have addressed the role of *O*-GlcNAcase inhibition beyond the use of PUGNAc. Thus, taking into account the off-target effects of PUGNAc, another approach, i.e., the RNA interference method, to reduce expression of *O*-GlcNAcase was used. Both methods have been similarly effective in increasing the *O*-GlcNAc level in cellular proteins.

The present study revealed an increased Akt1 activation in IGF-1-stimulated anaplastic thyroid cells and that down-regulation of *O*-GlcNAcase activity enhanced the effect of IGF-1 treatment. This study provides the first evidence that accumulation of *O*-GlcNAc in cells treated with PUGNAc or RNAi against *O*-GlcNAcase causes an increase in the phosphorylation of Akt1 Ser473. These observations were surprising since we expected rather a decrease than an increase in Akt phosphorylation. Many proteins, such as estrogen receptor β (Ser16), c-Myc (Thr58) and endothelial nitric oxide synthase

(Ser1177), have been shown to be reciprocally *O*-GlcNAc modified or phosphorylated (17). Reciprocal modification can also occur between adjacent residues. *O*-GlcNAc modification at Ser149 of p53 decreases the phosphorylation at Thr155 thereby stabilizing p53 by blocking ubiquitin-dependent proteolysis. Based on the reciprocal occupancy of *O*-GlcNAc and *O*-phosphate on several well-characterized proteins, a 'yin-yang' model has been proposed. This model hypothesizes that *O*-GlcNAcylation and phosphorylation modulate protein functions, in part, by competitively blocking each other's occupancy at given sites. There are literature data showing *O*-GlcNAcylation of Akt1 in different cells treated with PUGNAc. Treatment of murine pancreatic β cells with glucosamine was associated by *O*-GlcNAc modification of Akt1 and concominant reduction in Ser473 phosphorylation (18). Another study showed that in SH-SY5Y neuroblastoma cells treated with IGF-1, Akt1 may be simultaneously modified by *O*-GlcNAc and phosphorylated but PUGNAc-mediated accumulation of Akt1 *O*-GlcNAc modification did not attenuate Akt1 phosphorylation (13). We were unable to detect *O*-GlcNAc moieties on Akt1 in the control nor in the PUGNAc- or RNAi-treated 8305C cells (data not shown). We suggest that this kinase is not *O*-GlcNAcylated in 8305C cells or that it may be *O*-GlcNAcylated on a very low level and the changes observed in its activation depend on other factors.

Wang *et al* (19) demonstrated that the relationship between *O*-GlcNAcylation and phosphorylation is more complicated than previously thought. In NIH-3T3 cells, an increase in *O*-GlcNAcylation levels by *O*-GlcNAcase inhibitor treatment resulted in lower phosphorylation at many sites on studied phosphopeptides and concomitantly caused increased phosphorylation at many other sites. These findings suggest that *O*-GlcNAcylation and phosphorylation are not only coordinated at the protein substrate level but also regulate each other's cycling enzymes.

Our previous studies concerning *O*-GlcNAcylation in thyroid lesions demonstrated increased activity of *O*-GlcNAcase in cancers in comparison to non-neoplastic cells (20). Thus, we expected that an increase in *O*-GlcNAcylation would be disadvantageous for anaplastic cancer cells. The results of the present study revealed the unexpected finding that down-regulation of *O*-GlcNAcase enhanced Akt1 activity and cell proliferation rather than suppressing it. Although these results suggest the important role of *O*-GlcNAc modification in signaling pathways in thyroid cancer cells, further studies are necessary to ascertain the cause of increased activation of Akt1 in 8305C cells treated with PUGNAc and RNAi. Other studies have also focused on the effect of *O*-GlcNAcylation on cancer cell behavior. Recently, Caldwell *et al* (21) revealed that reduction of *O*-GlcNAcylation through RNA interference of *O*-GlcNAc transferase in breast cancer cells leads to inhibition of tumor growth both *in vitro* and *in vivo* and is associated with decreased cell-cycle progression.

Mitogenic activity of IGF-1 has been previously demonstrated in thyroid cells. In FRTL cells IGF-1 can promote cell cycle progression via up-regulation of cyclin D_1 expression through the PI3K signaling pathway (10). IGF-1 signaling appears to be increased in thyroid carcinomas in comparison to normal tissue (22). IGF-1 and IGF-2 are often overproduced by tumor cells and act as stimulators of malignant cell division through binding and stimulating the activity of the IGF receptor (IGF-1R) (23). The IGF-1R level has been found to be consistently higher in thyroid tumors than in normal thyroid tissue. Blocking IGF-1R with a specific antibody (A12) was found to block Akt phosphorylation and decrease proliferation of anaplastic thyroid cancer cells (24). Our results confirmed the mitogenic activity of IGF-1 in 8305C cells and showed that reduced *O*-GlcNAcase activity enhances its effect. Although PI3K/Akt signaling is best known for mediating cell survival, accumulating evidence supports its additional role in cell proliferation by regulating G1/S progression. Activated Akt can promote cell proliferation by increasing the cyclin D_1 level (22). It has been shown that increases in the cyclin D_1 level in response to stimuli are mediated by different mechanisms, such as up-regulation of gene transcription, acceleration of translation and stabilization of its protein. Akt prevents cyclin D_1 ubiquitin-mediated degradation by inhibiting glycogen synthase kinase (GSK3β) activity (22). To test the possibility that IGF-1-stimulated Akt1 in 8305C cells promotes cell proliferation by inhibiting degradation of cyclin D_1, cells were incubated for 24 h with IGF-1 and then the cyclin D_1 level was assessed. The level of cyclin D_1 was higher in IGF-1-stimulated cells with down-regulated *O*-GlcNAcase activity than in the control cells. The level of phosphorylated GSK3β was increased as well. These results suggest that at least, in part, the increased proliferation of 8305C cells treated with PUGNAc and RNAi may depend on the IGF-1-Akt1-GSK3β-cyclin D_1 pathway.

To conclude, IGF-1 increases proliferation of 8305C cells via Akt1 activation, and down-regulation of *O*-GlcNAcase enhances this effect. We suggest that an increased *O*-GlcNAc level in 8305C cells affects the IGF-1-regulated cyclin D_1 level through enhanced activation of Akt1. Akt1 can promote cyclin D_1 protein stability by inhibiting GSK3β.

Further investigation of the modification role of *O*-GlcNAc in abnormal signaling pathways in anaplastic thyroid cancer cells may aid in designing and developing therapeutic reagents against this disease.

Acknowledgements

This research was supported by grant no. 301463534 from the Ministry of Science and Higher Education of Poland.

References

1. Pollak MN, Schernhammer ES and Hankinson SE: Insulin-like growth factors and neoplasia. Nat Rev Cancer 4: 505-518, 2004.
2. Sachdev D and Yee D: Disrupting insulin-like growth factor signaling as a potential cancer therapy. Mol Cancer Ther 6: 1-12, 2007.
3. Ciampolillo A, De Tullio C, Perlino E and Maiorano E: The IGF-1 axis in thyroid carcinoma. Curr Pharm Des 13: 729-735, 2007.
4. Chakravarty G, Santillan AA, Galer C, Adams HP, El-Naggar AK, Jasser SA, Mohsin S, Mondal D, Clayman GL and Myers JN: Phosphorylated insulin like growth factor-1 receptor expression and its clinico-pathological significance in histologic subtypes of human thyroid cancer. Exp Biol Med 234: 372-386, 2009.
5. Copeland RJ, Bullen JW and Hart GW: Cross-talk between GlcNAcylation and phosphorylation: roles in insulin resistance and glucose toxicity. Am J Physiol Endocrinol Metab 295: 17-28, 2008.

6. Butkinaree C, Park K and Hart GW: O-linked beta-N-acetylglucosamine (O-GlcNAc): extensive crosstalk with phospho rylation to regulate signaling and transcription in response to nutrients and stress. Biochim Biophys Acta 1800: 96-106, 2010.
7. Hanover JA, Krause MW and Love DC: The hexosamine signaling pathway: O-GlcNAc cycling in feast or famine, Biochim Biophys Acta 1800: 80-95, 2010.
8. Lazarus BD, Love DC and Hanover JA: O-GlcNAc cycling: implications for neurodegenerative disorders. Int J Biochem Cell Biol 41: 2134-2146, 2009.
9. Macauley MS and Vocadlo DJ: Increasing O-GlcNAc levels: an overview of small-molecule inhibitors of O-GlcNAcase. Biochim Biophys Acta 1800: 107-121, 2010.
10. Ren M, Zhong X, Ma CY, Sun Y, Guan QB, Cui B, Guo J, Wang H, Gao L and Zhao JJ: Insulin-like growth factor-1 promotes cell cycle progression via upregulation of cyclin D1 expression through the phosphatidylinositol 3-kinase/nuclear factor-kappaB signaling pathway in FRTL thyroid cells. Acta Pharmacol Sin 30: 113-119, 2009.
11. Jones JI and Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16: 3-34, 1995.
12. Park SY, Ryu J and Lee W: O-GlcNAc modification on IRS-1 and Akt2 by PUGNAc inhibits their phosphorylation and induces insulin resistance in rat primary adipocytes. Exp Cell Mol Med 37: 220-229, 2005.
13. Gandy JC, Rountree AE and Bijur GN: Akt1 is dynamically modified with O-GlcNAc following treatments with PUGNAc and insulin-like growth factor-1. FEBS Lett 580: 3051-3058, 2006.
14. Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, Kudlow JE, Michell RH, Olefsky JM, Field SJ and Evans RM: Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. Nature 451: 964-969, 2008.
15. Akimoto Y, Hart GW, Hirano H and Kawakami H: O-GlcNAc modification of nucleocytoplasmic proteins and diabetes. Med Mol Morphol 38: 84-91, 2005.
16. Haltiwanger RS, Grove K and Philipsberg GA: Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-beta-N-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-gluco pyranosylidene) amino-N-phenylcarbamate. J Biol Chem 273: 3611-3617, 1998.
17. Zachara NE and Hart GW: Cell signaling, the essential role of O-GlcNAc. Biochim Biophys Acta 1761: 599-617, 2006.
18. Kang ES, Han D, Park J, Kwak TK, Oh MA, Lee SA, Choi S, Park ZY, Kim Y and Lee JW: O-GlcNAc modulation at Akt1 Ser473 correlates with apoptosis of murine pancreatic beta cells. Exp Cell Res 314: 2238-2248, 2008.
19. Wang Z, Gucek M and Hart GW: Cross-talk between GlcNAcylation and phosphorylation: site-specific phosphorylation dynamics in response to globally elevated O-GlcNAc. Proc Natl Acad Sci USA 105: 13793-13798, 2008.
20. Krzeslak A, Pomorski L and Lipinska A: Elevation of nucleocytoplasmic beta-N-acetylglucosaminidase (O-GlcNAcase) activity in thyroid cancers. Int J Mol Med 25: 643-648, 2010.
21. Caldwell SA, Jackson SR, Shahriari KS, Lynch TP, Sethi G, Walker S, Vosseller K and Reginato MJ: Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene 29: 2831-2842, 2010.
22. Grimberg A: Mechanisms by which IGF-1 may promote cancer. Cancer Biol Ther 2: 630-635, 2003.
23. Pavelić J, Matijević T and Knezević J: Biological and physiological aspects of action of insulin-like growth factor peptide family. Indian J Med Res 125: 511-522, 2007.
24. Wang Z, Chakravarty G, Kim S, Yazici YD, Younes MN, Jasser SA, Santillan AA, Bucana CD, El-Naggar AK and Myers JN: Growth-inhibitory effects of human anti-insulin-like growth factor-I receptor antibody (A12) in an orthotopic nude mouse model of anaplastic thyroid carcinoma. Clin Cancer Res 12: 4755-4765, 2006.