Original Paper

N-Acetyl-Glucosamine Sensitizes Non-Small Cell Lung Cancer Cells to TRAIL-Induced Apoptosis by Activating Death Receptor 5

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Key Words

N-acetyl-glucosamine • TRAIL • Non-small cell lung cancer • Death receptor

Abstract

Background/Aims: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential anti-cancer agent due to its selective toxicity. However, many human non-small cell lung cancer (NSCLC) cells are partially resistant to TRAIL, thereby limiting its clinical application. Therefore, there is a need for the development of novel adjuvant therapeutic agents to be used in combination with TRAIL. **Methods:** In this study, the effect of N-acetyl-glucosamine (GlcNAc), a type of monosaccharide derived from chitosan, combined with TRAIL was evaluated *in vitro* and *in vivo*. Thirty NSCLC clinical samples were used to detect the expression of death receptor (DR) 4 and 5. After GlcNAc and TRAIL co-treatment, DR expression was determined by real-time PCR and western blotting. Cycloheximide was used to detect the protein half-life to further understand the correlation between GlcNAc and the metabolic rate of DR. Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to detect receptor clustering, and the localization of DR was visualized by immunofluorescence under a confocal microscope. Furthermore, a co-immunoprecipitation assay was performed to analyze the formation of death-inducing signaling complex (DISC). O-linked glycan expression levels were evaluated following DR5 overexpression and RNA interference mediated knockdown. **Results:** We found that the clinical samples expressed higher levels of DR5 than DR4, and GlcNAc co-treatment improved the effect of TRAIL-induced apoptosis by activating DR5 accumulation and clustering, which in turn recruited the apoptosis-initiating protease caspase-8 to form DISC, and initiated apoptosis. Furthermore, GlcNAc promoted

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DR5 clustering by improving its O-glycosylation. **Conclusion:** These results uncovered the molecular mechanism by which GlcNAc sensitizes cancer cells to TRAIL-induced apoptosis, thereby highlighting a novel effective agent for TRAIL-mediated NSCLC-targeted therapy.

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Published by S. Karger AG, Basel

Introduction

Lung cancer is a major cause of cancer-related deaths worldwide, accounting for over 1 million deaths each year [1]. More than 80% of lung cancers are classified as non-small cell lung cancers (NSCLCs). At present, there is no substantive treatment breakthrough, and the 5-year survival rate is still less than 15%. Other than surgery, chemotherapy and radiotherapy are the main treatments for early lung cancer, which can non-specifically kill both cancerous and healthy cells [2]. In order to avoid damaging normal cells, tumor-targeted drugs have been of particular interest among medical researchers [3, 4].

Molecular targeting therapy has been recently developed for lung cancer. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising therapeutic agent used for tumor targeting therapy because it can selectively induce apoptosis in many cancers [5, 6]. However, nearly half of NSCLCs are resistant to TRAIL-induced apoptosis, which has limited its clinical application [7, 8]. In some cases, TRAIL resistance has been induced by overexpression of anti-apoptotic molecules, such as cellular FLICE inhibitory protein (FLIP); however, none of these factors have been consistently correlated with TRAIL resistance in multiple cancer cells [9]. FLIP expression levels were not correlated with susceptibility to TRAIL in 6 NSCLC cell lines [10]. Thus, it is very important to elucidate the molecular mechanisms of TRAIL resistance in NSCLC cells, and identify promising approaches that might enhance the sensitivity of these cells to TRAIL-induced apoptosis.

TRAIL is a member of the TNF family [11, 12]. TRAIL can induce the clustering of death receptor (DR) 4 or 5 on the targeted cell surface, triggering the formation of death-inducing signaling complex (DISC) at the inner surface of the plasma membrane [13-15]. Within DISC, receptors with apoptosis-inducing domains (death domains) bind the adaptor molecule, Fas-associated death domain (FADD), and recruit the apoptosis-initiating protease procaspase-8, which is auto-catalytically cleaved, releasing active caspase-8 into the cytoplasm to cleave and activate its effectors, caspase-3 and -7 [16, 17]. Thus, TRAIL-mediated DR activation is a crucial upstream event for triggering DISC assembly to initiate TRAIL-induced apoptosis.

However, the expression and native distribution of DRs on the cell surface were not related to the sensitivity of NSCLC cells to TRAIL. Instead, the determining factor is the functional status of the receptors, which is more important than the overall expression levels. Receptor clustering and redistribution on the plasma membrane is an important characteristic of its functional activation and is closely related to TRAIL sensitivity [18]. By comparing 2 types of lung cancer cell lines (H460: TRAIL-sensitive; A549: TRAIL-resistant), researchers found that DR redistribution contributed to the differences in TRAIL sensitivity [9]. Some drugs or interventions are able to improve TRAIL sensitivity by improving the functional status of DRs [19-21]. Thus, TRAIL receptor targeting therapy may be a promising approach for NSCLC treatment; however, there remains a need to identify agents that can activate DRs to improve sensitivity to TRAIL and broaden its application. We found that DR5 was expressed more highly than DR4 across 30 NSCLC tumor samples, suggesting that it may play a major role in TRAIL-induced apoptosis.

O-linked glycans regulate the biochemical and functional properties of cell surface proteins, including conformation, multimerization, trafficking, and turnover. A mechanism that modulates TRAIL signaling in tumors through DR O-glycosylation was previously discovered [22]. There are 2 forms of protein O-glycosylation: the first is initiated by N-acetyl-galactosamine (GalNAc) at serine or threonine residues [23], and the other is mediated by N-acetyl-glucosamine (GlcNAc), which is also an important regulatory mechanism in cell physiology [24]. O-glycan biosynthesis involves the activity of glycosyltransferases and glycosidases. GalNAc transferase (GALNT), mediating post-translational O-glycosylation of

DRs, is associated with receptor clustering efficiency and TRAIL sensitivity in many types of cancer cells, including NSCLC cells [22]. However, little is known about the effect of O-GlcNAcylation on the receptors.

GlcNAc is the degradation product of chitosan, the second most abundant polysaccharide in nature extracted from shrimp, crab, insect, and fungal exoskeletons. It has been shown that chitosan has many biological activities, including antimicrobial, antitumor, and immune-enhancing activities [25, 26]. However, its application has been greatly limited because of its water-insoluble characteristics. Oligochitosan and monosaccharides have recently been of interest because, in addition to being water-soluble, biocompatible, biodegradable, and non-toxic, they also possess versatile functional properties [27, 28]. GlcNAc is involved in a series of physiological regulatory activities as a precursor of O-GlcNAcylation, a major regulatory protein modification mechanism in cell physiology [24, 29, 30].

In the present study, we examined the effect of GlcNAc on the TRAIL-resistant NSCLC cell line, A549. Our results showed that GlcNAc improved TRAIL-induced apoptosis by increasing DR5 clustering. Furthermore, GlcNAc improved O-glycosylation of DR5, which promoted its function. Hence, we identified a novel molecular mechanism of GlcNAc-sensitized TRAIL-induced apoptosis, thereby highlighting a potentially effective agent for targeted therapy in TRAIL-resistant NSCLC.

Materials and Methods

Materials and reagents

Nude mice were purchased from the Central Laboratory for Experimental Animals of Nanjing (Nanjing, China). GlcNAc was prepared in our laboratory. Lung cancer cell lines (A549 and H460) were provided by the Typical Culture Preservation Commission Cell Bank, Chinese Academy Of Sciences (TCHu150, TCHu205). The normal lung cell line HELF was provided by the Central Laboratory of Affiliated Hospital of Qingdao University. 3-(4, 5-dimethylthiazol-2-yl)-2, 5, diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC/propidium iodide (PI) Staining Kit and Caspase-8 Activity Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Materials for cell culture, including RPMI-1640 culture medium and fetal bovine serum (FBS), were purchased from Gibco Co. (Grand Island, NY, USA). All other reagents used were of reagent grade.

Methods

MTT assay

H460, A549, and HELF cell lines were maintained in RPMI-1640 culture medium supplemented with 10% FBS. When the cells reached logarithmic growth phase, they were seeded in 96-well culture plates at an optimal density (5×10^3 cells per well) in a CO₂ (5%) incubator at 37°C. After a 24 h incubation period, the culture medium was removed. Triplicate wells were treated with TRAIL (50 ng/mL). Cellular responses were examined with the MTT assay by measuring absorbance at 490 nm after 24, 48, and 72 h, and cell morphology was analyzed by microscopy on day 1. A549 cells were cultured with GlcNAc (2 mM), TRAIL (50 ng/mL), or GlcNAc (2 mM)+TRAIL (50 ng/mL). Cells cultured in medium alone served as the control. On days 2, 4, and 7, cellular responses were assessed using the MTT assay. Experiments were performed in triplicate. Cell growth curves were calculated as the mean values of each group. This treatment dose was used for all further cell culture experiments.

Flow cytometry, caspase-8 activity analysis, and fluorescence microscopy for apoptosis detection

To further determine the number of apoptotic cells, Annexin V assays were performed using an apoptosis detection kit. Briefly, H460 and A549 cells were cultured in 6-well plates overnight. The medium was then removed, and cells were treated with GlcNAc, TRAIL, or GlcNAc+TRAIL. After 4 h, cells were harvested, washed in cold phosphate buffered saline (PBS), incubated for 15 min with fluorescein-conjugated Annexin V and PI, and analyzed using a FACS caliber (BD). At the same time, cells were collected and their lysate was used for caspase-8 activity detection. After culturing for 24 h, A549 cells were stained using the same assay

and analyzed by fluorescence microscopy. Control cells cultured with normal medium were stained with both PI and Annexin V.

DT-mediated dUTP nick-end labeling (TUNEL) assay to detect cell apoptosis

To measure apoptosis with the TUNEL assay, we used the FragEL™ DNA Fragmentation Detection Kit (Merck, Germany). A549 cells were cultured on glass slides and incubated with GlcNAc, TRAIL, or GlcNAc+TRAIL for 24 h. Cells cultured in normal medium served as the control. Following this, slides with cells were washed gently, fixed in 4% paraformaldehyde for 30 min, and stained as per the manufacturer's instructions. The TUNEL-positive cells were examined and counted using a light microscope (Olympus IX73). The apoptotic ratio was determined by measuring the number of TUNEL-positive cells out of 300 total cells. The experiment was performed in triplicate.

Anti-tumor effects in an in vivo xenograft model

Five-week-old nude mice, half male and half female, were allowed to acclimatize to the surroundings for 1 week before the experiments. Each mouse was injected under its arm with A549 cells (2×10^6). When tumors reached a size of approximately $5 \times 5 \text{ mm}^2$, mice were arbitrarily assigned to 4 groups to receive intraperitoneal injections: (1) vehicle alone (control), (2) GlcNAc alone, (3) TRAIL alone, and (4) GlcNAc+TRAIL. GlcNAc and TRAIL prepared in PBS (pH 7.4) were administered at 50 and 2.5 mg/kg/d, respectively. Mice received an intraperitoneal injection once every 2 days. The tumor size was measured every 5 days regularly using a Vernier's caliper to measure 2 perpendicular diameters, and the tumor size was calculated using the following equation: $(\text{length} \times \text{width}^2)/2$. After 20 days of injections, all animals were sacrificed. The tumor tissue, liver, spleen, and kidney of each animal were stripped and kept intact, and then weighed. Liver, spleen, and kidney indices were measured as the ratio of the organ weight to the body weight. Tumors were embedded in OCT compound (Miles Inc., Elkhart, IN, USA) and sliced into 5 μm sections using a cryostat (SLEE International, Inc., New York, NY, USA). Apoptotic cells were detected using the TUNEL assay.

Western blot analysis

A549 cells were cultured overnight in tissue plates and treated with 2 mM GlcNAc or 50 ng/mL TRAIL. The cells were harvested and extracted with modified RIPA buffer supplemented with phosphatase inhibitor cocktail (Roche). Protein extracts were boiled in sample buffer containing sodium dodecyl sulfate (SDS)/ β -mercaptoethanol. Purified proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes, which were subsequently blocked for 1 h in 5% fat-free milk in TBS-T, and then incubated overnight with anti-human primary antibody at 4°C. The following day, the blots were incubated with the appropriate HRP-conjugated secondary antibodies (111-035-045; 115-035-062; Jackson Immuno Research Laboratories, Inc.), and specific binding was detected using an ECL kit (Pierce, Appleton) with Vilber FX6. Additionally, the proteins were treated with sample buffer without β -mercaptoethanol for non-reducing SDS-PAGE to detect DR clustering.

The anti-human primary antibodies used include: DR4 (#42533), DR5 (#8074), caspase-8 (#9746), PARP (#9542), O-GlcNAcylation transferase (OGT; #5368) (Cell Signaling Technology, Beverly, MA, USA), and GAPDH (G9545; Sigma-Aldrich).

Quantitative real-time PCR (RT-PCR)

Total RNA of cells and tumor tissues was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). cDNA was prepared using reverse transcriptase (TaKaRa), and then used as a template with SYBR Green PCR master mix (TaKaRa). The following primers were used for the amplification of human DR4, DR5, and actin: DR4: (sense) 5'-TGC AGG TCG TAC CTA GCT CAG-3', (antisense) 5'-ACA TGC TGT GTT CCT GGT CGT-3'; DR5: (sense) 5'-CAA GAC CCT TGT GCT CGT TGT-3', (antisense) 5'-GGA GGT CAT TCC AGT GAG TGC-3'; and actin: (sense) 5'-GGC ATC GTC ACC AAC TGG GAC-3', (anti-sense) 5'-CGA TTT CCC GCT CGG CCG TGG-3'. Quantitative RT-PCR was performed using Roche 480 with the following parameters: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) values were calculated for all of the genes present.

Cycloheximide (CHX) chase experiments

Cells on plates were cultured with 10 µg/mL CHX (Sigma-Aldrich) for 30 min. The culture medium was then replaced with media supplemented with 10 µg/mL CHX alone as the control, or with CHX (10 µg/mL)-GlcNAc (2 mM), CHX (10 µg/mL)-TRAIL (50 ng/mL), or CHX (10 µg/mL)-TRAIL (50 ng/mL)-GlcNAc (2 mM) for the experimental treatments. Proteins were collected and extracted at different time intervals for western blot analysis, which was performed with a rabbit anti-DR5 antibody (CST; 1:1000 dilution), and the bound antibody was detected using HRP-conjugated goat anti-rabbit secondary IgGs. Detection by chemiluminescence reaction was performed using the ECL kit (Pierce). GAPDH (Sigma-Aldrich) was used as a loading control. Three independent experiments were performed.

Immunofluorescence observation by confocal microscopy

Cells were cultured on glass slides to 60–70% confluence, and then treated with TRAIL or GlcNAc. Firstly, cells were fixed with 4% PBS-buffered paraformaldehyde for 20 min, washed with PBS 3 times, and blocked with 5% bovine serum albumin for 1 h. Following this, cells were incubated with DR5 rabbit antibody at 4°C overnight, and then counterstained with FITC-conjugated goat anti-rabbit secondary antibody (CST; MA, USA) in the dark for 1 h. DAPI was used to stain the cell nuclei. Immunofluorescence results were analyzed using a confocal laser scanning microscope (Leica, Wetzlar, Germany).

Protein complex co-immunoprecipitation (co-IP)

To detect ligand-induced high molecular mass receptor complexes by co-IP, 1×10^7 cells were stimulated with TRAIL or GlcNAc for 1 h. Cells were harvested in lysis buffer, including 50 mM Tris-HCl, 130 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF, 10 mM NaF, 1% Triton X-100, and complete protease inhibitor (Roche). DR5 or normal IgG antibody was incubated with 1 mg cell lysate and 20 µL protein A/G agarose beads (Pierce) overnight at 4°C. The bound proteins were eluted off the beads using the elution buffer, and were further analyzed by western blotting. PVDF membranes were incubated overnight with mouse caspase-8 (CST) antibody at 4°C, and then incubated with the appropriate HRP-conjugated or conformation-specific (L27A9) HRP-conjugated (#5127; CST) secondary antibodies on the following day. Specific binding was detected using the ECL kit (Pierce) with Vilber FX6. DR5 on the PVDF membrane was detected using the same assay as described above. Total protein (30 µg) was analyzed by western blotting using antibodies against DR5 (#8074), and GAPDH (G9545; Sigma-Aldrich) was used as a loading control.

Recombinant protein preparation and DR5 O-glycosylation detection

DR5 was subcloned into PiggyBac vector as previously described [31], and the overexpression plasmid was named PiggyBac-DR5 (pDR5). A549 cells cultured on the plates were transfected with pDR5 using Lip3000 (Invitrogen) for 24 h, and then stimulated with TRAIL or GlcNAc for 8 h. Cells were harvested in lysis buffer and prepared for western blotting to detect DR5 or OGT.

OGT or DR5 interference

Two to three sequences were designed and prepared for DR5 or OGT interference, respectively. A549 cells were cultured on 24-well tissue plates overnight, and OGT or DR5 expression was knocked down by RNA interference (RNAi). After 48 h, total RNA of cells was isolated using TRIzol reagent for quantitative PCR to compare and select the sequence with the highest interference efficiency. Then, A549 cells were cultured again, and OGT or DR5 knockdown was carried out with the screened RNAi sequences. After 48 h, the medium was replaced with GlcNAc-, TRAIL-, or GlcNAc+TRAIL-supplemented media and cultured for 1 h. Next, cells were harvested for western blotting to detect the protein expression of DR5 (#8074), caspase-8 (#9746), PARP (#9542), and OGT (#5368) (Cell Signaling Technology, Beverly, MA, USA). Protein expression was normalized to that of GAPDH (G9545; Sigma-Aldrich).

Statistical analysis

Individual experiments were performed at least in duplicate and repeated a minimum of 3 times. Statistical analyses are accompanied by graphs prepared using Prism software (GraphPad, La Jolla, CA, USA). The results of western blot analysis were normalized to GAPDH protein expression. Bar graphs represent mean $\pm$ standard deviation (SD). Results were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Fig. 1. Comparing apoptosis induced in lung cancer (H460 and A549 cell line) and normal (HEL F) cell lines. Relative growth rate were detected by MTT assay from 24 to 72 h (A) and morphological images at 24h were showed (B). Apoptosis cells of A549 and H460 were detected by Flow cytometry after treating with TRAIL and/or GlcNAc (C&D) for 4h and corresponding death or apoptosis cells were calculated (E&F). At the same time, caspase 8 activities were detected (G). The experiments were carried out independently in triplicate. Each bar shows the mean $\pm$ SD. * p <0.05: significantly different, ** p <0.01: highly significantly different.

Results

Effects of TRAIL on lung cancer and normal cells

Sensitivity of the lung cancer cell lines, H460 and A549, to TRAIL was compared with that of normal cell line, HELF. When H460 cells were incubated with TRAIL (50 ng/mL), most cells underwent apoptosis between 24 to 72 h, and their relative growth rates were much lower than those of A549 or HELF cells (Fig. 1A). Morphological images also indicated that H460 cells were round in shape at 24 h after TRAIL treatment, whereas only a few HELF and A549 cells were round in shape (Fig. 1B). These results indicated that H460 cells were sensitive to TRAIL, while A549 cells were insensitive.

Addition of GlcNAc elevating TRAIL sensitivity in lung cancer cells

Although A549 lung cancer cells are resistant to TRAIL treatment, they become sensitized when TRAIL is combined with other agents [32]. In the present study, we examined the effects of GlcNAc in combination with TRAIL. Cell apoptosis was analyzed using flow cytometry after treatment with TRAIL alone or in combination with GlcNAc. A549 and H460 cells were exposed to 50 ng/mL TRAIL and/or 2 mM GlcNAc for 4 h, and then analyzed using both Annexin V and PI assays. The apoptotic rate of TRAIL-treated A549 cells was roughly 25%, and significantly increased to 40% when co-treated with GlcNAc (Fig. 1C, E). A high percentage of apoptotic H460 cells was observed after TRAIL treatment, and more numbers of cells were found to be apoptotic following GlcNAc+TRAIL co-treatment (Fig. 1D, F). These data also showed that H460 cells were more sensitive than A549 cells, and that addition of GlcNAc could elevate TRAIL sensitivity, consistent with the results from caspase-8 activity detection (Fig. 1G).

Effects of GlcNAc and TRAIL on A549 cell apoptosis

Based on the above results, studying the role of GlcNAc and TRAIL in insensitive cancer cells could be much more meaningful than in sensitive cancer cells for tumor treatment. To further confirm their combinatorial effects on A549 cells, multiple assays were performed. First, when A549 cells were incubated with both GlcNAc (2 mM) and TRAIL (50 ng/mL), most of the cells underwent apoptosis in a time-dependent manner, and there was no

Fig. 2. GlcNAc sensitizes

TRAIL-induced apoptosis of

NSCLC cell line A549.

A: The cytotoxic effects of

TRAIL and GlcNAc alone

or in combination on A549

cells. After treatment for

24 h, 48 h and 72 h, cell

viability was evaluated us-

ing the MTT assay. **B:** A549

cells were cultured with

TRAIL (50 ng/mL) and/

or GlcNAc (2 mM) for 36h.

Cells stained with Annex-

inV / PI were observed by

fluorescence microscope.

Cells cultured with 10%

FBS complete medium as

the control (100×). **C-D:**

A549 cells were cultured

on glass slides and incu-

bated with TRAIL and/or

GlcNAc for 36 h. Cells were

fixed for TUNEL staining

(C) (200×); (a) control: non-

treated cells, (b) GlcNAc-

treated cells, (c) TRAIL-

treated cells, (d) combina-

tion TRAIL and GlcNAc-

treated cells, (e) the nega-

tive control, (f) the positive

control. The rate of apopto-

sis was expressed as the per-

centage of total cells counted

(D). Each bar shows the

mean±SD of six independ-

ent experiments. A dark

brown DAB signal indicates

positive staining, while

shade of blue signifies a

non-reactive cell. The ex-

periments were carried

out independently in tri-

plicate; representative data

are shown. Sensitization

of TRAIL-induced apopto-

sis is improved by GlcNAc

through analyzing PARP

and death receptors. The

total and cleaved PARP

protein under different

treatment conditions (E)

and quantification of

cleaved PARP was nor-

malized to GAPDH.

There is significant

difference for the co-

treatment compared

with the group of

significant difference in absorbance from 24 to 72 h. After treatment with TRAIL and GlcNAc for 48 and 72 h, there were significantly fewer live cells as compared to treatment with TRAIL alone or the control (Fig. 2A). Furthermore, to verify whether TRAIL and GlcNAc are cytotoxic or induce A549 cell apoptosis, in addition to flow cytometry, morphological and biochemical assays were performed. A549 cells were incubated with 50 ng/mL TRAIL and/or 2 mM GlcNAc for 24 h, and then analyzed by fluorescent staining and TUNEL assay. Cells stained with Annexin V/PI were analyzed by fluorescence microscopy (Fig. 2B). With GlcNAc+TRAIL co-treatment, the number of apoptotic (green) and dead (red) cells resulting from TRAIL only treatment was increased. TUNEL results showed that A549 cells treated with GlcNAc or TRAIL alone had only slight incidence of spontaneous apoptosis (5.8 and 17.8%, respectively). In contrast, there were more number of apoptotic cells after combined treatment as compared with other groups, and the apoptotic ratio increased to 56.7% (Fig. 2C, D) ($P < 0.01$). These data demonstrate that the inhibitory effects of TRAIL and GlcNAc on cancer cells result from inducing cell apoptosis.

* $p < 0.05$: significantly different, ** $p < 0.01$: highly significantly different.

Examining the effect of apoptosis by analyzing the protein level of cleaved PARP

To further evaluate the effect of apoptosis induced by combined treatment with TRAIL and GlcNAc, cleavage of the executioner caspase substrate PARP was analyzed by western blotting (Fig. 2E). Quantitative analysis showed that the levels of cleaved PARP increased following co-treatment with TRAIL and GlcNAc, as compared to treatment with TRAIL alone (Fig. 2F) ($P < 0.01$).

Fig. 3. GlcNAc and TRAIL combining therapy induces apoptosis and necrosis of tumor. The mice received an intraperitoneal injection one time every two days. The tumor size was measured and calculated every 5 days regularly (A). After injection for 20 days, all the animals were sacrificed. The tumors were collected (B) and weighted (C). To detect the tumor apoptosis after treatment with GlcNAc and TRAIL, the apoptotic cells are detected by TUNEL assay and observed under light microscope (D, 200×); (a) vehicle alone as the control, (b) GlcNAc-treated alone, (c) TRAIL-treated alone, (d) combination TRAIL and GlcNAc-treated, (e) the negative control, (f) the positive control, and apoptosis cells for TUNEL positive staining in combined treatment group are indicated by arrows. Liver, spleen and kidney tissues were isolated completely and weighed to calculate index (E). More than three mice were prepared as mean±SD for each group. (* p<0.05, ** p<0.01).

Fig. 4. DR4 and DR5 expression in 30 tumor samples of NSCLC were detected (A). DR5 was expressed more highly than DR4 (p<0.01). Cells treated with TRAIL or GlcNAc were regularly collected for analysis by Western blotting to evaluate the level of DR4 (B) and DR5 (D). The levels of GAPDH served as the loading control for quantification. For the DR4 protein level, there are no significant difference between the treatment groups and the control (B, C). In the groups of treating with GlcNAc, DR5 protein level is up-regulated obviously (D, E). Transcription analysis of death receptors showed that there were no differences between the treated groups and the control at 4, 24 and 36h (F). Data are the means of triplicate determinations from three independent experiments. * p<0.05: significantly different, ** p<0.01: highly significantly different from the control.

GlcNAc increased TRAIL sensitivity of lung cancer xenografts in vivo

Four groups of nude mice received intraperitoneal injections once every 2 days of either: (1) vehicle alone (control), (2) GlcNAc alone, (3) TRAIL alone, or (4) GlcNAc+TRAIL. Tumor size was measured every 5 days

by measuring 2 perpendicular diameters. After 10 days, there were significant differences in tumor size between the 4 groups. Tumors in the control group grew to 5201 mm³, whereas those in the GlcNAc+TRAIL group grew to 1038 mm³ on day 20 (Fig. 3A). Similar results were obtained when the tumors were collected and weighed after treatment for 20 days. Our results demonstrated that A549 xenograft growth in athymic nude mice was greatly suppressed by combined treatment with GlcNAc and TRAIL, whereas the suppression of tumor growth was not significant in mice treated with GlcNAc or TRAIL alone (Fig. 3B, C). TUNEL assay showed that GlcNAc+TRAIL co-treatment largely induced A549 tumor cell death *in vivo* (Fig. 3D). In contrast, tumor cells in mice treated with GlcNAc or TRAIL alone had very few incidences of apoptosis. Thus, GlcNAc+TRAIL co-treatment effectively sensitized TRAIL-resistant lung cancer cells. Next, the liver, spleen, and kidney tissues were isolated completely and weighed to calculate indices (Fig. 3E). We found no significant difference in liver and spleen indices between the experimental and control groups ($P > 0.05$). These findings suggest that GlcNAc can sensitize NSCLC cells to TRAIL treatment *in vivo* with no apparent organ toxicity.

GlcNAc sensitizes A549 cells to TRAIL-mediated apoptosis by upregulating DR5 protein levels

The TRAIL receptors DR4 and DR5, which contain functional death domains, are crucial for apoptosis initiation because they trigger apoptotic signals upon TRAIL binding. Interestingly, DR5 was expressed more highly than DR4 across 30 NSCLC clinical samples (Fig. 4A; $P < 0.01$), suggesting that DR5 may play a more important role in TRAIL-induced apoptosis than DR4. To study the mechanism by which GlcNAc sensitizes A549 cells to TRAIL-mediated apoptosis, we assessed the protein levels of both DR4 and DR5. The DR4 protein level in GlcNAc+TRAIL-treated cells increased after 20 h, but was not significantly different from that

Fig. 5. The metabolic rate of DR4 (A-E) and DR5 (F-J) was analyzed by treating with GlcNAc or TRAIL. After treating with CHX, the western blot experiments are used to detect the protein levels of groups of control, GlcNAc, TRAIL and GlcNAc+TRAIL. Quantification protein was normalized by GAPDH to show the changed half-lives of DR4 (E) and DR5 (J). Although the half-life of DR4 was not detected within 24h, there were no obviously differences for all the groups at 24h. Treating with GlcNAc, the half-life of DR5 could be prolonged. In the group of CHX-TRAIL+GlcNAc, DR5 half-life is prolonged to over 2.5 h, which is much longer than the control and TRAIL groups. And it isn't detected within 6 h for CHX-GlcNAc group. Data were prepared as mean±SD for each group from three experiments.

in control cells ($P=0.0596772$). The protein level of DR4 did not change after treatment with TRAIL or GlcNAc alone (Fig. 4B, C). However, DR5 protein level increased with GlcNAc treatment from 4 to 36 h (Fig. 4D, E; $P < 0.01$ compared to control). DR5 protein expression levels were significantly different between the combined treatment and control groups at 20 and 36 h ($P < 0.01$). However, following treatment with TRAIL alone, there were no changes in protein levels after 36 h. For transcriptional analysis of the DRs, total RNA was isolated from A549 cells at 4, 24, and 36 h. Results showed that the relative mRNA levels of DR4 and DR5 were all less than 1.5-fold of those in the control group (Fig. 4F). These results indicate that GlcNAc sensitized A549 cells to TRAIL-mediated apoptosis by upregulating DR5 at the protein level, and not at the transcription level.

GlcNAc promotes DR5 protein accumulation by reducing its metabolic rate

In molecular biology and pharmaceutical research, CHX can be used to determine the half-life of the protein *in vitro* because it inhibits eukaryotic protein synthesis. After treatment with CHX, cell lysates were prepared at the indicated time points and equivalent amounts of total protein of DR4 (Fig. 5A–D) and DR5 (Fig. 5F–I) were analyzed. Fig. 5E and J show the change in half-life of DR4 and DR5 following treatment with GlcNAc and/or TRAIL. GlcNAc treatment prolonged the half-life of DR5. After CHX treatment, the half-life was measured to be approximately 0.8 h. The half-life increased to 1.1 h following co-treatment with CHX and TRAIL. However, combined treatment with CHX, TRAIL, and GlcNAc increased the DR5 half-life to over 2.5 h. Furthermore, it is worth noting that the half-life of DR5 was not detected within 6 h of observation following combined treatment with CHX and GlcNAc, whereas the half-life of DR4 was not detected within 24 h. Although DR4 protein levels following GlcNAc treatment seemed to be higher than those in other treatment groups at all time points up to the 8 h mark, there were no apparent differences between all treatment groups at 24 h. These results suggest that GlcNAc can change the half-life of DR5 and reduce its metabolic rate to promote protein accumulation.

GlcNAc promotes TRAIL-induced DR5 clustering

To further understand how GlcNAc reduces metabolic rate, we analyzed receptor clustering, which is critical for TRAIL-mediated DISC formation to initiate apoptosis.

Fig. 6. GlcNAc sensitizes TRAIL-mediated apoptosis of A549 cells by improving DR5 clustering. DR5 is significantly clustered by treating with GlcNAc or TRAIL+GlcNAc comparing with the control from 4h to 24h (A, B). The clustering DR4 is not detected in 24 h for all groups (C). To further investigate the distribution of DR5, the localization of death receptor induced by TRAIL could be observed by immunofluorescence under confocal microscopy (D). The arrows show that the main aggregates located in both the cytoplasm and cell membrane in the treating groups of TRAIL+GlcNAc. Data were the means of triplicate determinations from three independent experiments. * $p < 0.05$: significantly different, and ** $p < 0.01$: highly significantly different from the control.

Fig. 7. Analyzing formation of the death-inducing signaling complex (DISC). A549 cells were cultured with TRAIL or GlcNAc for 1 h, collected and lysed for IP with DR5 antibody and IB with caspase-8 antibody (A). The amount of Caspase8 was improved highly in the combined effect group (B) and DR5 protein of GlcNAc and combining treatment groups was significantly increased comparing with the TRAIL treated only and the control (C). The input protein was detected with antibodies of DR5 and GAPDH as a loading control (D). Data were the means of triplicate determinations from three independent experiments. ** $p < 0.01$: highly significantly different; * $p < 0.05$: significantly different from the control.

Members of the TNF receptor (TNFR) family, including DR4 and DR5, have extracellular cysteine-rich regions and intracellular death domains [33, 34]. When the receptors cluster, disulfide bridges form between the connected monomers, and the clustered receptors can be detected by non-reducing SDS-PAGE [22].

After treatment with GlcNAc or GlcNAc+TRAIL, a significant increase in DR5 clustering was observed from 4 to 24 h compared to control, and the maximum amount of aggregation was found at the 8 h mark (Fig. 6A, B). TRAIL only treatment induced substantially lesser DR5 clustering as compared with other treatments. In addition, DR4 clustering was not detected after 24 h for all treatments, despite an increase in exposure time (Fig. 6C). These results show that GlcNAc sensitizes A549 cells to TRAIL-mediated apoptosis by specifically promoting DR5 clustering. To further investigate the distribution of DR5, TRAIL-induced localization of DRs was visualized by immunofluorescence using a confocal microscope. Following control, TRAIL, and/or GlcNAc treatment for 8 h, A549 cells were collected. Immunofluorescent staining of DR5 revealed receptor clusters in GlcNAc and GlcNAc+TRAIL treatment groups. The main aggregates were located in the cytoplasm after GlcNAc treatment, whereas GlcNAc+TRAIL co-treatment induced clustering both in the cytoplasm and on the cell membrane. However, no clustering was observed in the control and TRAIL only treatment groups (Fig. 6D). Thus, combined treatment with TRAIL and GlcNAc resulted in DR5 clustering and redistribution on the cell membrane, which demonstrates that GlcNAc promotes DR5 protein clustering.

GlcNAc enhances TRAIL-induced DISC formation

DISC assembly at the inner surface of the plasma membrane is necessary for TRAIL-induced apoptosis. Normally, TRAIL-resistant cells have defective stimulation of caspase-8 processing and have weak DISC-mediated recruitment of DRs and caspase-8 [35]. To detect ligand-induced high molecular mass receptor complexes, cells were stimulated with normal medium, TRAIL, GlcNAc, or GlcNAc+TRAIL for 1 h, and a co-IP assay was performed with DR5 and caspase-8 antibodies. Our results indicated that DR5 protein was significantly clustered following GlcNAc treatment. Western blot analysis revealed an increased amount of cleaved caspase-8 following GlcNAc+TRAIL co-treatment compared to treatment with TRAIL alone (Fig. 7A–C). Proteins were detected with antibodies targeting DR5, and GAPDH was used as a loading control (Fig. 7D). Western blot analysis also showed that GlcNAc could improve DR5 clustering after one hour of treatment. In conclusion, these results showed that DISC formation was improved by treating A549 cells with a combination of TRAIL and GlcNAc.

Fig. 8. O-glycosylation level induced by TRAIL or GlcNAc was analyzed. After A549 cells were transfected with DR5-overexpression plasmid (pDR5) (A) for 24 h and then cultured with TRAIL or GlcNAc for 8 h, DR5 and OGT protein expressions were detected by Western blot (B). DR5 protein has been improved significantly by treating with TRAIL+GlcNAc (C), and OGT protein is elevated significantly by GlcNAc or TRAIL+GlcNAc treating (D). To detect GlcNAc effecting on O-GlcNAcylation of DR5, interference sequences were designed and prepared respectively for OGT (E) or DR5 (F). The most effective siRNAs targeting gene transcripts (OGT-1421, DR5-866) were screened by quantitative PCR in A549 cells. A549 cells were interfered by OGT or DR5 siRNA for 48 h, and then treated with GlcNAc, TRAIL or GlcNAc+TRAIL respectively for 1 h. OGT, PARP, Caspase 8 and DR5 protein level were showed (G–K). Data were the means of triplicate determinations from three independent experiments. * $p < 0.05$: significantly different, ** $p < 0.01$: highly significantly different from the control. The potential molecular mechanism of GlcNAc sensitizing cancer cells to TRAIL-induced apoptosis was shown (L).

GlcNAc enhancing O-GlcNAcylation of DR5

O-linked glycans can regulate the functional properties of cell surface proteins. O-glycan biosynthesis involves the activity of glycosyltransferases and glycosidases. O-GlcNAcylation, a major regulatory mechanism in cell physiology, is mediated by OGT. Given that DR5 was more strongly expressed than DR4 in 30 NSCLC tumor samples (Fig. 4A), and that GlcNAc affected DR5 more than DR4, equal amounts of DR5-overexpressing plasmid (Fig. 8A) were transfected into A549 cells for 24 h. The cells were then treated with normal medium, TRAIL, GlcNAc, or GlcNAc+TRAIL for 8 h in order to investigate the correlation between DR5 O-GlcNAcylation and TRAIL sensitivity. Western blot analysis revealed that DR5 protein levels increased significantly following co-treatment with GlcNAc+TRAIL (Fig. 8B, C), and OGT protein levels also increased significantly following GlcNAc and GlcNAc+TRAIL treatments (Fig. 8B, D).

To detect the effect of GlcNAc on DR5 O-GlcNAcylation, effective siRNAs targeting OGT or DR5 gene transcripts (OGT-1421, DR5-866) were screened with A549 cells, and more than half mounts of mRNA were interfered (Fig. 8E, F). A549 cells were treated with screened siRNAs for 48 h, and then treated with normal medium, GlcNAc, TRAIL, or GlcNAc+TRAIL for 1 h. Western blotting analysis revealed that OGT and DR5 were effectively knocked down by siRNA, and there were significant differences between the normal control and corresponding RNAi groups (Fig. 8G, H, K). Following TRAIL only and GlcNAc+TRAIL treatments, OGT, cleaved PARP, and caspase-8 expression levels were reduced when DR5 was knocked down, and cleaved PARP and caspase-8 expression was also inhibited when OGT was knocked down by RNAi (Fig. 8G–J). This demonstrates that O-glycosylation of DR5 is important for TRAIL-induced apoptosis. In cells that were not treated with siRNAs, cleaved PARP and caspase-8 levels were much higher following GlcNAc+TRAIL co-treatment than those following TRAIL treatment alone. However, there were no obvious differences in cleaved PARP and caspase-8 levels between the 2 groups when OGT or DR5 was knocked down by RNAi (Fig. 8G, I, J), suggesting that increased apoptosis induced by GlcNAc+TRAIL co-treatment is strongly related to DR5 and its O-glycosylation.

These results showed that GlcNAc can improve the expression of OGT to enhance O-glycosylation of DR5, resulting in DR5 clustering and cell sensitization to TRAIL-induced apoptosis. The putative molecular mechanism by which GlcNAc sensitizes cancer cells to TRAIL-induced apoptosis is shown in Fig. 8L.

Discussion

More than 80% of lung cancers are classified as NSCLCs. Chemotherapy and radiotherapy affect both cancerous and healthy cells. In order to avoid damaging normal cells, therapeutic strategies to specifically target tumor cells are needed.

TRAIL is a very promising agent for targeted cancer therapy because it can selectively induce apoptosis in cancer cells, with little or no toxicity toward normal cells [36]. However, studies have shown that many types of cancers, including some NSCLCs, are resistant to the apoptotic effects of TRAIL [32, 37–39]. It has been reported that absence of DR expression and clustering may be a major cause of resistance to TRAIL-based cancer therapy [18]. Thus, it is important to develop agents that can sensitize cells to TRAIL-induced apoptosis by restoring TRAIL receptor expression and clustering to form DISC and initiate apoptosis.

On comparing the effect of TRAIL on cancer (A549 and H460) and normal cell lines (HELFI), A549 cells were found to be resistant to TRAIL-induced apoptosis (Fig. 1). A549 cells co-treated with GlcNAc and TRAIL triggered a synergistic apoptotic response *in vitro* (Fig. 2) and *in vivo* (Fig. 3). *In vitro*, there were no apparent differences following GlcNAc and control treatments with respect to cell activity, PARP cleavage, and caspase-8 expression. GlcNAc increased DR5 clustering, but could not trigger apoptosis. However, *in vivo*, GlcNAc triggered apoptosis as determined by TUNEL staining. Further studies are needed to determine the other pathways, through which apoptosis was induced. Immune responses were likely not responsible for this effect because nude mice were used. GlcNAc sensitized A549 cells to TRAIL-mediated apoptosis by upregulating DR5 (and not DR4) expression at the protein level, but not at the transcriptional level (Fig. 4), by changing the half-life of DR5 and reducing its metabolic rate (Fig. 5). DRs have extracellular cysteine-rich regions and intracellular death domains [33, 34]. When the receptors cluster, disulfide bridges form between the connected monomers. Receptor clustering can be detected by non-reducing SDS-PAGE. DR5 formed clusters on the cell membrane following GlcNAc+TRAIL co-treatment (Fig. 6), which likely results in reduced degradation and increased receptor expression.

Receptor clustering-induced DISC formation is very important for TRAIL-induced apoptosis [40]. Upon TRAIL activation, DRs on the targeted cell surfaces cluster, and DISC is assembled at the inner surface of the plasma membrane [39]. Only clustered receptors on the cell surface can promote DISC formation and initiate apoptosis [41–43]. TRAIL-resistant cells

have defective stimulation of caspase-8 processing and exhibit weaker receptor-mediated DISC formation and caspase-8 recruitment [35]. There was a substantial increase in caspase-8 cleavage following GlcNAc+TRAIL co-treatment compared to TRAIL only treatment (Fig. 7), consistent with enhanced DISC assembly. However, individual proteins within DISC, such as TNF receptor type 1-associated death domain (TRADD) and FADD, were difficult to detect by co-IP because their molecular weight was close to that of light chain of IgG.

Chitosan is derived from chitin, which is the second most abundant biopolymer in the exoskeleton of crustaceans, insects, and fungi. Its applications are limited because of its water-insoluble characteristics. Hence, recent studies have focused on oligochitosan and monosaccharides [44-46]. GlcNAc is a degradation product of chitosan, and is not only water-soluble, biocompatible, biodegradable, and non-toxic, but also possesses versatile functional properties [27, 28]. GlcNAc is an important monosaccharide in cells, and may be involved in physiological regulation. The covalent attachment of GlcNAc to serine/threonine residues in proteins is a major regulatory mechanism in cell physiology [29, 30]. In relevant studies, we found that GlcNAc could be linked to TRAIL because the melting temperature (T_m) of TRAIL was changed in the presence of GlcNAc, as determined by differential scanning fluorimetry [47], which is a rapid and inexpensive screening method to identify low-molecular-weight ligands that bind and stabilize purified proteins. If a compound binds to a protein, the free energy contribution of ligand binding in most cases results in an increase in Gibbs free energy of unfolding, which may cause an increase in the T_m . The T_m of TRAIL was 52.45°C, which could be elevated to 53.74°C with GlcNAc treatment. This showed that GlcNAc could bind to the TRAIL protein.

In the present study, we found a novel function of GlcNAc in improving DR5 clustering and promoting TRAIL-induced DISC formation to initiate apoptosis. We also described a putative mechanism. This novel effect may be associated with O-GlcNAcylation of DRs. Previous findings suggest that specific O-glycosylation might be involved in post-translational modification of apoptosis components because of highly conserved O-glycosylation sites in DRs. This finding has been useful in identifying patients with cancer who are more likely to respond to TRAIL-based therapies. Sensitivity of cancer cells to TRAIL-induced apoptosis is modulated by promoting ligand-induced receptor clustering, which is associated with efficient DISC recruitment and caspase-8 activation [48, 49]. The ability of GlcNAc to increase DR5 clustering maybe related to the receptor's O-glycosylation level. In fact, DR5 was more strongly expressed than DR4 across 30 NSCLC tumor samples, suggesting that DR5 may play a major role in TRAIL-induced apoptosis (Fig. 4A). In order to detect the correlation between DR5 O-GlcNAcylation and TRAIL sensitivity, overexpression and knockdown experiments were performed. We found that OGT protein levels were significantly increased when cells were co-treated with TRAIL and GlcNAc, consistent with increased receptor O-glycosylation (Fig. 8A-D). Furthermore, not only was apoptosis suppressed, but there were no obvious differences between TRAIL only and GlcNAc+TRAIL treatments when OGT and DR5 were knocked down (Fig. 8G-K). Therefore, the mechanism by which GlcNAc sensitizes cells to TRAIL-induced apoptosis could be related to the O-glycosylation level of DR5.

Although DR5 and DR4 are both DRs of TRAIL, they can act through different mechanisms. It has been reported that persistent endoplasmic reticulum (ER) stress causes accumulation of intracellular DR5, triggering DR5 activation and inducing apoptosis via caspase-8; however, DR4, Fas, and TNFR1 expression was only weakly affected [50]. In addition, the sarcoplasmic ER calcium-adenosine triphosphatase inhibitor thapsigargin and the glycosylation inhibitor tunicamycin, which regulate DR5 and not DR4, enhanced cell sensitivity to TRAIL. Therefore, although DR4 and DR5 can form S-S linked oligomers, they can be regulated by different complex mechanisms. Moreover, post-translational modifications mediated by O-glycans are complex, and their specific location and initiating monosaccharides are associated with several different mechanisms. For example, GalNAcylation at serine or threonine residues [23] and GlcNAcylation on a conserved arginine residue [51] can lead to inverse cellular responses. Further studies identifying new mechanisms would be of great value.

Conclusion

In summary, we found that DR5 was highly expressed in NSCLC tumor samples, suggesting that it plays an important role in TRAIL-induced apoptosis. Importantly, we found that GlcNAc enhanced TRAIL-induced apoptosis in NSCLC cells by accelerating DR5 clustering and accumulation, and by promoting its O-glycosylation, which is necessary for efficient DISC recruitment, caspase-8 activation, and subsequent initiation of apoptosis. In conclusion, GlcNAc may serve as a promising agent to sensitize cancer cells to TRAIL-induced apoptosis.

Acknowledgements

This study was financially supported by the National Natural Science Foundations of China (81401899, 81472411, 81772713, 81372752, 81672662); Taishan Scholar Program of Shandong Province (tsqn20161077); China Postdoctoral Science Foundation (2017M622144) and Qingdao Postdoctoral Application Research Project; Qingdao Young Scientist Applied Basic Research Fund (15-9-1-51-jch, 16-6-2-28-NSH); Natural Science Foundation (ZR2014HM088, ZR2014CM040), Key Research and Development Program of Shandong Province (2017GSF18193) and the Science and Technology Development Foundation (2014GHY115025) of Shandong Province.

Disclosure Statement

The authors have no conflicts of interest to declare.

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