Glucosylated heparin derivatives as non-toxic anti-cancer drugs

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Abstract

Heparin, which has been widely used as an anti-coagulant agent, has potential anti-tumor effects; in particular, low molecular weight heparin (LMWH) may inhibit tumor angiogenesis and/or metastasis with reduced toxicity. For decades, it has been known that malignant cancer cells display abnormally enhanced glucose uptake rates and overexpress glucose transporters (GLUTs) compared to normal cells. With these findings in mind, we introduced a glucose moiety to heparin for the purpose of increasing the concentration of heparin at the tumor site by targeting GLUTs. Three glucosylated heparin (GH) derivatives were prepared by conjugation of glucosamine and heparin in different mole ratios. To evaluate the potential of GH derivatives as anti-cancer drugs, their anti-coagulant activities, inhibitory effects on glucose analog uptake, cellular interactions, tumor growth inhibitory effects and sub-acute toxicities were investigated. The anti-coagulant activities of GH derivatives decreased proportionally to the degree of glucosylation. In vitro, GH derivatives inhibited HUVEC proliferation to a greater extent than heparin. GH derivatives mainly existed outside of cells and interacted with GLUTs on the cell surface, thereby inhibiting glucose uptake into cells. In vivo, GH derivatives significantly suppressed tumor growth compared to control, without systemic toxicity. Therefore, GH derivatives are proposed as potent non-toxic anti-cancer drugs.

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Keywords: Heparin; LMWH; GLUTs; Glucosylated heparin; Anti-cancer drug

1. Introduction

Heparan sulfate proteoglycans (HSPGs) are reported to play important roles during tumor progression [1–5]. Heparin, widely used as an anti-coagulant agent in the clinic for the past 60 years, is a highly sulfated member of the HSPG family. By competing with HSPGs, heparin can inhibit tumor-cell adhesion, heparanase activity and binding of HSPGs and growth factors. Heparin can also activate the attack by NK cells in the immune system, thereby inhibiting tumor angiogenesis and metastasis [6–10]. Several clinical evidences indicate that heparin has a marked effect on tumor metastasis and angiogenesis. Chemically modified heparin was recently developed to reduce the side effects of heparin given at therapeutic levels for cancer treatment [11–14]. It has been reported that heparin derivatives modified with bile acids, such as deoxycholate and lithocholate, exerted a significant anti-tumor effect by inhibiting angiogenesis as nano-sized particles [15–19].

Tumor cells are dependent on glycolysis to support their metabolic requirements. Even under aerobic conditions, tumor cells continue to rely on glycolysis rather than oxidative phosphorylation (Warburg effect), resulting in high glucose requirements to generate energy and support metabolic function [20,21]. Many kinds of human cancers such as breast cancer, colorectal cancer, gastric cancer, head and neck cancer, lung cancer, ovarian cancer, pancreatic cancer, thyroid cancer, and...
bladder cancer, express elevated levels of glucose transporters (GLUTs) compared to normal tissues [22–24]. High GLUT1 expression tends to correlate with tumors having high proliferative and metastatic activity and poor prognosis [25–28]. Thus, increased glucose uptake and high expression of GLUTs may represent an important regulatory point in the maintenance of growth and synthesis activity of malignant cells and suppression of apoptosis [29–32]. In fact, abnormally increased glucose uptake by tumors or overexpression of GLUTs in tumors is viewed as a promising target for cancer diagnosis and treatment. In the clinical setting, glucose uptake can be visualized in many tumors in vivo using positron emission tomography (PET) [33–35]. Many researchers have developed and used glucosamine [36,37] and 2-deoxyglucose [38] as glucose analogs clinically, finding that they distribute selectively to tumors and inhibit glycolysis.

Based on the important role of heparin in cancer and the high expression of GLUTs in cancer cells, we designed water-soluble glucosylated heparin (GH) derivatives, i.e., heparin modified with glucosamine, which can target the GLUTs of cancer cells. The aim of this study was to develop an effective and non-toxic cancer therapeutic agent using heparin and glucosamine, which are natural, non-toxic materials. We expected that GH derivatives could act as glucose uptake blockers in cancer cells, thereby causing the starvation of cancer cells. Three GH derivatives were prepared by conjugation of glucosamine and heparin in different mole ratios. The anti-coagulant activity of the GH derivatives was determined. Also, FDG uptake inhibition and cellular interaction were examined, along with tumor growth inhibition and sub-acute toxicity.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (LMWH), with an average molecular weight ca. 4500 Da (Fraxiparin, 97 IU/mg), was purchased from GlaxoSmithKline (GlaxoSmithKline UK Ltd., Middlesex, UK). Glucosamine, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC), ethylenediamine, fluorescein isothiocyanate (FITC) and 0.2% ninhydrin spray reagent were purchased from Roche Applied Science (Penzberg, Germany) and Sigma-Aldrich, respectively. All buffer solution components were analytical grade, and all aqueous solutions were prepared exclusively in deionized distilled water.

2.2. Synthesis of GH derivatives

GH derivatives were synthesized by coupling heparin with glucosamine, a glucose analog. Three GH derivatives with different glucosamine content were prepared by adjusting the mole ratios of heparin:EDAC:glucosamine as 1:1:1, 1:2:2 or 1:3:3, respectively. In brief, 200 mg of heparin was dissolved in 10 ml of distilled water and 8.52 mg, 17.04 mg or 25.56 mg of EDAC was then added, according to the mole ratio. Then 9.58 mg, 19.16 mg or 28.74 mg of glucosamine was added to the heparin solutions, respectively. The pH was adjusted to 5.0 with 1 N HCl solution. After stirring for 12 h at room temperature (RT), the reaction mixture was dialyzed (MWCO 2000) and lyophilized. The glucosamine content in GH derivatives was determined by the ninhydrin assay: Amount of reacted glucosamine was determined by subtracting the amount of unreacted glucosamine in reaction mixture from the initial feed amount and then coupling ratio of heparin and glucosamine was calculated from these values. Briefly, 100 μl of the reaction mixture was taken prior to dialysis and mixed with 100 μl of ninhydrin solution (0.2%). The sample was heated at 100 °C for 3 min, cooled down immediately in ice bath, and absorbance from the sample was analyzed at 570 nm. A calibration curve was constructed using different concentrations of glucosamine (15–250 μg/ml) in water. And the anti-coagulant activities of GH derivatives were evaluated using anti-FXa chromogenic assay (COATEST®Heparin, Milano, Italy).

FITC-labeled heparin and FITC-labeled GH derivatives were prepared for in vitro experiments. Prior to FITC labeling, amine groups were introduced to the heparin and GH derivatives by reacting with them with ethylenediamine of 100-fold excess. FITC was then conjugated to the aminated heparin and aminated GH derivatives.

2.3. MTT assay

The cytotoxicity of GH derivatives to murine colon adenocarcinoma cells (CT 26 cells) was evaluated using the MTT assay. The CT 26 cell line was purchased from Korean Cell Line Bank (KCLB, Korea) and maintained at 37 °C and 5% CO2 atmosphere in DMEM supplemented with 3.7 g of sodium bicarbonate, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 1% antibiotic-antimycotic, and 10% fetal bovine serum. The cells were cultured to 70–80% confluence, harvested with trypsin-EDTA and washed twice with serum-free culture media. CT 26 cells were seeded at 5 × 104 cells/well in 96-well flat-bottomed culture plates in supplemented DMEM and cultured for 12 h at 37 °C and 5% CO2 atmosphere. Cells were then incubated with serial dilutions of GH derivatives (0.2–100 μM) for 24 h in quadruplicate and labeled by adding 20 μl of sterile-filtered MTT solution (5 mg/ml in PBS) to determine metabolic activity. After incubating cells at 37 °C for 4 h, the supernatants were carefully removed from the wells, and the formazan crystals were dissolved in DMSO. Absorbance was quantified using a microplate fluorescence reader (Synergy HT, BIO-TEK®) at 570 nm. Percent viabilities were
2.5. Inhibition of FDG uptake in vitro

370 nm.

Finally, the absorbance in CO2 atmosphere. The cells were harvested with trypsin-EDTA, and the radioactivity in the supernatant and multiplying by 100% ∼

expression as ratios of absorbance by heparin or GH derivative-
treated cells compared to control.

2.4. BrdU incorporation assay

The cell proliferative activity of GH derivatives in human umbilical vein endothelial cells (HUVECs) was investigated using the bromodeoxyuridine (BrdU) incorporation assay. HUVECs were purchased in passage 3 from Modern Cell & Tissue Technologies, Inc. (MCTT, Korea) and used in passages 5–8. HUVECs were grown in EBM®-2 supplemented with EGTM™-2 SingleQuots at 37 °C in a humidified atmosphere of 5% CO2. The cells were cultured to 70∼80% confluence, harvested with trypsin-EDTA and washed twice with serum-
free culture media. HUVECs were seeded at 5 × 10³ cells/well in EBM-2 in 96-well flat-bottomed culture plates and cultured for 12 h at 37 °C and 5% CO2 atmosphere. The cells were then incubated with various dilutions (1 ∼ 100 μM) of heparin or GH derivatives in culture media for 24 h at 37 °C and 5% CO2 atmosphere. The cell proliferation assay was then carried out using a BrdU assay kit purchased from Roche. Briefly, the drug-containing media was removed, and the cells were rinsed once with PBS. The cells were labeled with 100 μl/well of BrdU labeling agent for 10 h at 37 °C, and the labeling medium was removed by tapping. Next, the cells were incubated with 200 μl/well of fixative solution for 30 min at RT. After removal of fixative solution, 100 μl/well of Anti-BrdU-POD (peroxidase) working solution was added. The cells were incubated for 60 min and rinsed 3 times with washing solution at RT. After removal of washing solution by tapping, the cells were incubated with 100 μl/well of TMB (tetramethyl-benzidine) substrate solution for 30 min at RT. Finally, the absorbance in each well was measured using an ELISA plate reader at 370 nm.

2.5. Inhibition of FDG uptake in vitro

CT 26 cells were seeded at 1 × 10⁶ cells/well in DMEM in 6-
well culture plates and cultured overnight at 37 °C and 5% CO2 atmosphere. The medium was then exchanged with glucose-free DMEM, and the cells were incubated with 100 μM heparin or GH derivatives and 50 μCi/well of FDG for 2 h at 37 °C and 5% CO2 atmosphere. The cells were harvested with trypsin-EDTA, washed with media and centrifuged at 1200 rpm for 5 min at 4 °C. Radioactivity in the cell pellets and in 1 ml of supernatant was counted by a gamma counter (Cobra 5010 Detector, Packard Instrument Company). The amount of FDG taken up by the cells (%) was calculated by dividing the radioactivity in the cell pellet by the sum of the radioactivity in the cell pellet and the radioactivity in the supernatant and multiplying by 100%.

2.6. Cellular interaction (microscopic images)

CT 26 cells were seeded at a density of 4 × 10⁴ cells/well in 8-Chamber Slides™ (Lab-Tek™) and cultured overnight in DMEM containing 4.5 g/l of glucose. After changing the medium to DMEM containing 1.0 g/l of glucose, the cells were incubated with 100 μM FITC-labeled heparin or 100 μM FITC-labeled GH derivatives for 2 h at 37 °C and 5% CO2 atmosphere. The drug-containing media was then removed, and the cells were washed three times with cold PBS and fixed in 1% paraformaldehyde solution for 1 h on ice. The fixed cells were stained with TRITC-phalloidin (10 μM) and 4′,6-diamidino-2-phenyl indole (DAPI, 5 μg/ml) for 20 min and 10 min, respectively. Cell images were taken with Image Restoration Microscopy (DeltaVision RT, AppliedPrecision).

2.7. Inhibition of 2-NBDG accumulation in vivo

Colon adenocarcinoma-bearing Balb/c mice (7-week-old males, Orient Bio Co., Ltd., Korea) were prepared by inoculation with a suspension of 1 × 10⁶ CT 26 cells/100 μl into the shaved right dorsa as previously described [39]. The animals were housed under normal conditions with 12-h light and dark cycles and were given access to food and water ad libitum. All animal experiments were performed in accordance with procedures described in the Guide for the Care and Use of Laboratory Animals and the animal care and studies were approved by the Institute of Laboratory Animal Resource of the Seoul National University (Seoul, Korea). The CT 26 tumor-bearing mice were randomized into five experimental groups of 4 mice as follows: non-treated (control), heparin (+ control), and each of three GH derivatives. The heparin and GH derivatives were intravenously administered to mice at a dose of 1 mg/kg. In 5 min, i.v. injection of 10 mg/kg of 2-NBDG followed. At times of 5 and 15 min postinjection of 2-NBDG, tumor tissues were isolated, weighed and homogenized in 2 ml of lysis buffer per tumor weight (g). NBD fluorescence from the lysed sample was detected at excitation/emission of 465/540 nm using a microplate fluorescence reader. The amount of 2-NBDG accumulated in tumor tissue (μg/g) was calculated based on a calibration curve constructed using different concentrations of 2-NBDG.

2.8. Anti-tumor activity of GH derivatives in mice

The CT 26 tumor-bearing mice were randomized into five experimental groups of 7 mice as follows: non-treated (control), heparin, and each of three GH derivatives. Tumors were allowed to progress to a volume of 50 ∼ 60 mm³ before initiating treatment. The heparin and GH derivatives were intravenously administered to mice at a dose of 1 mg/kg every two days for two weeks. Tumor sizes were measured daily in two dimensions using a vernier caliper. Tumor volumes were calculated using the formula \( V = \) (length) ∗ (width²) / 2. Relative tumor volume % (RTV) was calculated for each individual tumor by dividing tumor volume by the corresponding tumor volume at the start of treatment (day 0) and multiplying by 100% \( \text{RTV} = \) (Tumor volume at day \( X \) / Tumor volume at day 0) ∗ 100. At the end of the experimental period, mice were sacrificed, and tumor tissues were isolated and weighed.
2.9. Sub-acute toxicity

Among the three GH derivatives, the sub-acute toxicity of GH2 was investigated at three different doses using ICR mice (5-week-old males, Orient Bio Co., Ltd., Korea). Normal ICR mice in groups of ten were administered 1, 10, or 50 mg/kg GH2 intravenously every two days seven times. The mice were carefully observed for physiological and behavioral responses and mortality, and their body weights were measured and recorded daily. At the end of the experimental period, selected organs (spleen, liver, lung, heart and kidney) were weighed, and blood samples were collected by retro-orbital puncture. Whole blood and serum were used for hematological and serological tests, respectively. In whole blood, the following hematological parameters were analyzed: white blood cell count (WBC), red blood cell count (RBC), platelet count, hemoglobin (Hb), hematocrit (Hct), segment, band, lymphocyte, monocyte, eosinophil, basophil, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and reticulocytes. To obtain serum, blood samples were placed at RT for 30 min. The tubes were then centrifuged at 3000 × g for 10 min at 4 °C, and supernatants were used for the following determinations: serum glucose, blood urea nitrogen (BUN), total protein, creatinine, globulin, albumin, total bilirubin, sodium, and potassium concentrations, as well as the enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH).

2.10. Statistical analysis

Statistical differences between each group with respect to tumor growth and weight were determined using the standard Student’s t-test. p values of <0.05 were considered significant.

3. Results

3.1. Synthesis and characterization of GH derivatives

According to the synthetic scheme illustrated in Fig. 1, glucosamine was conjugated to heparin in various mole ratios. The degree of glucosylation of GH1, GH2 and GH3 was 0.86, 1.49 and 2.92, respectively, meaning that GH2 contained 149 glucose molecules per 100 heparin molecules. These values represent an average value since heparin has a molecular weight distribution. The anti-coagulant activity of GH1, GH2 and GH3, as evaluated by anti-Factor Xa chromogenic assay, was 70.1 IU/mg, 23.8 IU/mg and 16.0 IU/mg, respectively. The anti-coagulant activity of GH derivatives decreased significantly as the degree of glucosylation increased. The characteristics of GH derivatives are listed in Table 1.

3.2. Effects of GH derivatives on viability of CT 26 cells and proliferation of HUVECs

Cytotoxicity of GH derivatives on CT 26 cells was evaluated by MTT assay. After 24 h incubation with heparin or GH derivatives, most cells were viable regardless of drug concentration (Fig. 2(a)), indicating that GH derivatives did not affect cell viability and neither did heparin. In BrdU incorporation assay, GH1 and GH2 significantly suppressed HUVEC proliferation, whereas heparin and GH3 did not affect the proliferation of HUVECs. At 100 μM, GH1 and GH2 potently inhibited HUVEC proliferation by 58.6% and 41.3%, respectively, compared to control, whereas GH3 and heparin suppressed the growth of HUVECs by 29.7% and 27.3%, respectively (Fig. 2(b)).

3.3. Cellular interaction of GH derivatives

In order to evaluate whether GH derivatives blocked GLUTs and inhibited glucose uptake into cells, FDG was used for uptake assays instead of glucose. FDG, 2-[18F]fluoro-2-deoxy-D-glucose, is a glucose analog and a promising imaging agent for positron emission tomography (PET). As shown in Fig. 3, the inhibitory effect of GH1, GH2 and GH3 on FDG uptake into cells was 44.7, 23.4 and 12.7%, respectively. GH1 had a much greater inhibitory effect than GH2 and GH3. The inhibitory

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mole ratio (heparin: EDAC:glucosamine)</th>
<th>Coupling ratio a (heparin:glucosamine)</th>
<th>Anti-coagulant activity b (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>1:1:1</td>
<td>1: 0.86±0.04</td>
<td>142.5 c</td>
</tr>
<tr>
<td>GH1</td>
<td>1:2:2</td>
<td>1: 1.49±0.16</td>
<td>70.1</td>
</tr>
<tr>
<td>GH2</td>
<td>1:3:3</td>
<td>1: 2.92±0.02</td>
<td>33.9</td>
</tr>
<tr>
<td>GH3</td>
<td>1:4:4</td>
<td>1: 3.92±0.04</td>
<td>16.0</td>
</tr>
</tbody>
</table>

a Measured by Ninhydrin assay.
b Evaluated by Factor Xa chromogenic assay.
c Evaluated after dialyzing heparin under the same conditions used during the synthesis of GH derivatives.
effect of GH3 on FDG uptake was the lowest and was similar to
that of heparin (13.9%).

After determining that GH derivatives interact with GLUTs
on the cell surface, the interactions between GH derivatives and
CT 26 cells were analyzed microscopically. Cells were exposed
to FITC-labeled heparin or FITC-labeled GH derivatives (green
color) in the medium with or without phloretin, a GLUT
inhibitor, and the cells were then double-stained with DAPI
(blue color) and TRITC-phalloidin (red color) to stain the
nucleus and to detect cell shape and junctions, respectively.
Cells treated with heparin and GH3 did not display any FITC
signal, regardless of phloretin treatment (Fig. 4a, b, g, and h).
On the other hand, cells treated with GH1 and GH2 exhibited
FITC signal and the FITC signal appeared around the cell
membrane when exposed to medium without phloretin (Fig. 4c
and e). And Cells in medium containing phloretin could not
interact with GH1 and GH2 and did not exhibit any fluor-
escence (Fig. 4d and f).

3.4. Inhibition of 2-NBDG uptake in tumor by GH derivatives

In vivo, inhibitory effect of GH derivatives on glucose uptake
in tumor was investigated. In this experiment, glucose uptake
in tumor was monitored using 2-NBDG, which is a fluorescent
glucose analog, with fluorescence detection. As shown in Fig. 5,
GH derivatives significantly inhibited the uptake of circulating
2-NBDG in tumor compared to heparin. At 5 min post injection,
the amount of 2-NGDG in tumor was lowest, followed in
ascending order by the GH2 group and the GH3 group, and they
showed the same tendency at 15 min post injection. That is,
GH1 had a much higher inhibitory effect than GH2 and GH3. In
both cases in vitro and in vivo, it was demonstrated that GH
derivatives interact with GLUTs and inhibit glucose uptake in
cancer cells and in tumor, respectively. There was no significant
difference between heparin group and control group at both
times.

3.5. Inhibitory effect of GH derivatives on tumor growth

As shown in Fig. 6(a), GH1 and GH2 effectively suppressed
tumor growth compared to control, whereas GH3 and heparin
did not statistically inhibit tumor growth. Compared to control,
GH1 and GH2 treatment resulted in 42.3% and 56.6% tumor
growth inhibition, respectively. The tumor growth inhibitory
effect of GH2 seemed greater than that of GH1, but there was no
statistically significant difference between their effects
\(p=0.816\). When we compared tumor weights in the various
treatment groups on the final day of the experiment, the results
agreed well with those based on tumor volumes (Fig. 6(b)).
Mean tumor weight in the GH2 group was lowest, followed in
ascending order by the GH1 group, the GH3 group, the heparin
group, and finally, by the control group. As with tumor volume
change, there was no statistically significant difference between
the tumor weight change of GH1 and GH2 (p=0.236). The inhibitory effect of GH derivatives on tumor growth was in accord with their anti-proliferative effect on HUVECs.

3.6. Sub-acute toxicity

GH2 was selected to evaluate the systemic toxicity of GH derivatives because it was the most effective tumor growth inhibitor. Intravenous administration (q2d × 7) of GH2 to mice at doses of 1, 10, and 50 mg/kg over a period of two weeks did not cause death, abnormal behavior or autonomic signs. During the two-week experimental period, the administration of GH2 did not affect weight gain in mice (Fig. 7(a)).

The hematological parameters of GH2-treated mice were not significantly different from those of control mice (Table 2). The number of RBCs and the hemoglobin level, which together indicate RBC balance, were measured to determine whether GH2 induced anemia by influencing RBCs. No significant change in the number of RBCs was observed in GH2-treated groups compared to control. The number of WBCs was within the permissible range. Also, the MCV, MCH, and MCHC levels, which are related to the condition of the RBCs, and the Hct value, which indicates the proportion of cells and fluids in blood, were unaltered versus control. One of the important factors related to heparin toxicity is platelet number, although LMWH has a lower risk of inducing thrombocytopenia than UFH. During GH2 treatment, the platelet count in the plasma remained in the normal range.

The serological parameters of GH2-treated mice were also similar to those of control mice (Table 3). The GH2-treated groups had similar reticulocyte concentrations compared to control. Serum glucose, BUN, creatinine, total protein, albumin, globulin, and total bilirubin concentrations and ion concentrations, such as sodium and potassium, as well as the activity of marker enzymes (AST, ALT, ALP, and LDH) in the GH2-treated groups were not significantly different from the control values. AST/ALT ratios, a sensitive indicator of hepatocyte damage, were not altered in the GH2-treated groups. These results indicate that GH2 is not toxic to mouse liver. Also, GH2 did not remarkably alter various biochemical levels, such as BUN, creatinine, albumin, globulin and total bilirubin. The content of inorganic ions, such as sodium and potassium, in the blood were measured to check for any alterations in biological balance induced by GH2 treatment. Consequently, the inorganic ion contents were similar to that of the control, indicating that GH2 did not change the balance of ions in the body, a change which can be induced by abnormal hormone control. To observe any changes in body weight, the weights of organs including the liver, heart, kidney, lung and spleen were measured. As shown in Fig. 7(b), no organ weight changes were detected in GH2-treated groups compared to control.

4. Discussion

The objective of this study was to evaluate glucosylated heparin in targeting and blocking cancer cells to result in improved therapeutic efficacy and reduced toxicity. Glucosamine was covalently bound to the carboxyl groups of low molecular weight heparin by amide bonds. The three GH derivatives contained 0.86, 1.49 and 2.92 glucosamine molecules per heparin molecule, respectively. The anti-coagulant activities of the GH derivatives significantly decreased with the increase in the degree of glucosylation. Even when about one glucosamine molecule is conjugated to one heparin molecule as in GH1, the anti-coagulant activity
decreases to 49.2% compared to that of heparin. Therefore, glucosylation can reduce the risk of bleeding, which is the most serious side effect of heparin.

Heparin binds to proteins such as proteases, growth factors, angiogenic factors, lipid-binding proteins or adhesion proteins in its function as an anti-coagulant agent or anti-cancer agent [40]. Clearly, the most prominent type of interactions between heparin and a protein is ionic interaction, as well as non-ionic interactions such as hydrogen bonding. This protein-binding is associated with the L-iduronic acid ring flexibility and the glucosidic torsions of the trisaccharide unit that allow a conformational change in the helical repeat pattern of the acidic and polyanionic biopolymer. It makes possible to fit between unique heparin sequences and the protein to which it specifically binds [41]. When modified inordinately, the heparin will undergo a conformational change and lack the functional groups that participate in protein-binding. This will nearly make heparin derivatives useless drugs, as the anti-coagulant activity is reduced to almost nothing. When modifying heparin, therefore, it is necessary to optimize the relationship between the anti-coagulant activity and anti-tumor activity of heparin derivatives to design the best cancer therapeutic.

In the evaluation of HUVEC proliferation and growth of murine CT 26 adenocarcinoma, among the three GH derivatives, GH1 and GH2 exhibit significantly superior inhibitory effects compared to heparin. Even though they have lower anti-coagulant activity than heparin itself, GH1 and GH2 show much greater inhibitory effects on HUVEC proliferation and murine adenocarcinoma growth than heparin. On the other hand, GH3 has very low anti-coagulant activity and does not effectively inhibit cell proliferation and tumor growth. When we consider the relationship of anti-coagulant activity and anti-proliferative and anti-tumor activities of heparin derivatives based on the results from these GH derivatives, glucosylation using two or fewer carboxyl groups, as in GH1 and GH2, decreases anti-coagulant activity of heparin but simultaneously increases the anti-proliferative and anti-cancer activities. That is, the conjugation of glucosamine to heparin enhances the anti-proliferative activity and anti-tumor activity of heparin. However, glucosylation of heparin using more than two carboxyl groups, as in GH3, deprives heparin of anti-coagulant, anti-proliferative, and anti-tumor activities. Therefore, if the heparin derivative loses excessive anti-coagulant activity, it may also be deprived its anti-proliferative and anti-tumor activities in the bargain.

In the FDG uptake inhibition and cellular interaction, at first, we would have expected that as glucosylation increase, the FDG uptake inhibition in cells and the interaction between GH derivatives and cells are more dominant. However, the increase
of the glucose content in the heparin derivatives decreased the FDG uptake inhibition. Just comparing GH1 and GH2, the FITC signal slightly increased as glucosylation increased, but GH3 did not display any FITC signal, regardless of phloretin treatment, evading our expectation definitely. We surmised that GH3 could not easily interact with cells due to the steric hindrance by the conjugated glucosamine compared to GH1 and GH2. It can explain some unexpected result in the FDG uptake inhibition. This FDG uptake inhibition in vitro was in accord with the 2-NBDG uptake inhibition in tumor in vivo. In both cases, GH1 exhibited highest inhibitory effect among GH derivatives and even GH3, which showed lowest inhibition effect in vitro, displayed considerable inhibition effect in vivo. From this result, we found out that GH derivatives might block the GLUTs and inhibit the glucose uptake into cells as a barrier against GLUTs of cancer cells, but the increase of glucose content in the GH derivatives does not certainly increase the FDG uptake inhibition. The interaction of GH derivatives and GLUTs primarily appears on the cell surface and seems to be reversible and weak. We can infer that GH derivatives are not able to induce apoptosis or cause side effects in normal cells or organs that express GLUTs. Based on FDG and 2-NBDG uptake inhibition and cellular interaction results, therefore, GH1 and GH2 may be targeted to tumor site by the conjugated glucosamine, interact with both endothelial cells and cancer cells, and influence the proliferation of endothelial cells and the glucose uptake of cancer cells, thereby exhibiting better anti-tumor activity than heparin.

Moreover, GH derivatives do not affect cell viability like heparin, but more significantly inhibit cell proliferation than heparin. When used in vivo, the anti-tumor effect of the GH derivatives was superior to that of heparin against CT 26 adenocarcinoma in Balb/c mice. In addition, compared with control (non-treated mice), GH2, which remarkably suppressed tumor growth, did not significantly alter body weight and organ weight of the liver, heart, kidney, lung and spleen that can be a sign to indicate the side effects by GH2. Regardless of dose, additionally, GH2 did not significantly change various hematological and serological parameters that indicate the organ health indirectly. Although WBC counts and differentiation values showed slight difference compared to control group after GH2 treatment, they were within the permissible range and monocyte level also was within the normal range (0.0 ~ 5.0%).

![Fig. 7. Sub-acute toxicity of GH2.](image)

**Table 2**

<table>
<thead>
<tr>
<th>Hematological parameters after 2-week GH2 treatment in mice (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>WBC (×10³/µl)</td>
</tr>
<tr>
<td>Segment (%)</td>
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<tr>
<td>Band (%)</td>
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<tr>
<td>Lymphocyte (%)</td>
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<td>RBC (×10³/µl)</td>
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<tr>
<td>Hb (g/dl)</td>
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<td>Hematocrit (Hct,%)</td>
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<td>MCV (fL)</td>
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<tr>
<td>MCH (pg)</td>
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<tr>
<td>MCHC (%)</td>
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<tr>
<td>Platelet (×10³/µl)</td>
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<tr>
<td>Reticulocyte (%)</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Serological parameters after 2-week GH2 treatment in mice (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Protein, total (g/dl)</td>
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<tr>
<td>Albumin (g/dl)</td>
</tr>
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<td>Globulin (g/dl)</td>
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<td>BUN (mg/dl)</td>
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<td>Creatinine (mg/dl)</td>
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<td>Na (mmol/l)</td>
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<td>K (mmol/l)</td>
</tr>
</tbody>
</table>
In this study, we evaluated the abilities of GH derivatives to target tumors and then block GLUTs on the surface of cancer cells, resulting in significant anti-tumor efficacy. We also investigated the systemic toxicity of GH derivatives to examine whether GH derivatives cause side effects in normal tissues, particularly in organs that express GLUTs. The results reported here demonstrate proof that GH derivatives have potential as anti-cancer drugs without side effects. GH derivatives, represented by GH2, are therefore systemically non-toxic and do not induce severe side effects against normal tissues and organs, including those that usually express GLUTs. From this study, GH derivatives may be desirable anti-cancer drugs that will target the tumor site without causing severe side effects like some of the current anti-cancer drugs.

5. Conclusion

By taking advantage of the role of heparin in cancer and abnormally increased glycolysis in cancer cells, we designed heparin modified with glucosamine and prepared GH derivatives with different glucosamine content. Compared to heparin, the anti-coagulant activities of GH derivatives decreased proportionally to the degree of glucosylation. GH derivatives remarkably inhibited HUVEC proliferation compared to heparin. The GH derivatives interacted with GLUTs on the cell surface and inhibited glucose uptake into cells. GH derivatives also significantly suppressed the growth of murine adenocarcinoma compared to control and heparin-treated groups, but were not systemically toxic during the treatment based on hematological and serological findings. From this study, GH derivatives hold promise as anti-cancer drugs with excellent anti-tumor effects but without systemic toxicity.

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References


