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Glycated chitosan as a new non-toxic immunological stimulant

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Abstract

Chitosan is capable to stimulate immune responses. However, because chitosan is not water soluble, it has limited biological applications. By attaching galactose molecules to the chitosan molecules, a new water-soluble compound, glycated chitosan (GC), was synthesized. GC was designed for immune stimulations in combination with phototherapies in the treatment of metastatic tumors. To investigate the possible toxicity of GC, cultures of normal and tumor cells were incubated with GC of different concentrations and the cell viabilities were determined. For *in vivo* studies, GC solution was fed or injected to animals and its toxicity was determined through observations of animals and histological examinations of vital organs. No toxic effects of GC were observed in cultured cells or in animal studies. In addition, the immunological effect of GC was investigated through its stimulation of TNF α secretion by macrophages *in vitro*. *In vivo* studies showed enhancement of the survival of laser immunotherapy-treated rats bearing metastatic mammary tumors. Our *in vitro* and *in vivo* results indicated that GC was a strong immunological stimulant. Its non-toxic nature and immunological activity make GC a potential immunoadjuvant for treatment of metastatic tumors.

Keywords

Chitosan; galactose; glycated chitosan; toxicity; TNF α ; macrophages; immune responses

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INTRODUCTION

Chitosan is a linear polysaccharide composed of randomly distributed β -(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is obtained by partial deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (1, 2). Chitosan has been used to treat fungal infections in plants (3, 4) and for water filtration (5, 6). Chitosan also has been used in a variety of biomedical applications: it serves as a blood clotting agent (7), a dietary supplement for weight loss (8–11), and an antibacterial agent (12), among others. Chitosan has been shown to be able to induce and enhance immunological responses (13–18).

Chitosan is relatively insoluble in water but is soluble in diluted acids (19, 20). The poor solubility of chitosan poses limitations for its biomedical applications. Especially in the areas of immunology, an aqueous solution is essential for its use as an immunostimulant in clinical applications. Glycated chitosan (GC) is a new compound derived from chitosan by attaching galactose molecules to the chitosan molecules (21). GC, while retaining the biological properties of chitosan, is water soluble, thus making it more suitable for *in vitro* and *in vivo* biomedical applications.

GC was first used as an immunoadjuvant for the treatment of experimental animal tumors in laser immunotherapy (22). Laser immunotherapy was developed to use a photothermal laser treatment in combination with an immunoadjuvant to treat metastatic tumors (23–26). Specifically, it uses a near-infrared laser and an intratumorally administered light-absorbing dye to achieve a selective photothermal interaction (27–29). GC was administered locally to induce a specific immunological reaction. This method has been used successfully in treating metastatic breast tumors in rats. Not only were the treated primary tumors successfully destroyed, but untreated metastatic tumors at remote sites were also eradicated. In addition, laser immunotherapy-cured rats could withstand subsequent repeated tumor challenges with increased tumor doses as well as provide protection to naive animals against the same tumor through adoptive immunity transfer using immune spleen cells (24). We have shown that GC is capable of inducing tumor-specific immune responses in the laser treated tumor-bearing animals at the cellular and molecular levels (23).

Based on the results of our pre-clinical studies, GC has a potential to serve as a potent immunostimulant for cancer treatment, particularly for metastatic tumors, when combined with other modalities of direct interventions, such as photothermal therapy (23–26) and photochemical therapy (30). The future clinical applications require a thorough understanding of the properties of GC. The work presented here is the first in a series of studies designed to understand the physical and biological properties of GC as well as its immunological mechanisms. Specifically, we briefly review the synthesis procedures of GC. We also present the preliminary results of *in vitro* and *in vivo* GC toxicity studies. Furthermore, we report the preliminary results of our studies on the immunological mechanisms of GC in activating macrophages as well as in treating tumors in combination with photothermal therapy.

MATERIALS AND METHODS

1. Synthesis of glycated chitosan

One gram of chitosan chloride (Pronova Biopolymer, Oslo, Norway) was added to 100 ml of deionized water in a beaker and stirred magnetically to obtain a homogeneous suspension. Then 3 grams of galactose was added slowly to the chitosan solution while stirring. Toluene, 0.25 ml, was added as a preservative. The beaker was covered with aluminum foil and the solution was stirred for 24 hours. Five milliliters of sodium borohydride solution (1.327 gram of sodium borohydride in 5 ml of 0.1 N NaOH) were added in 0.2 ml aliquots to avoid excessive foaming. The solution was then stirred for ten minutes at room temperature and for 50 minutes in an ice bath. Excess borohydride was decomposed with glacial acetic, again avoiding excess foaming. The final pH was set at 5.4 – 5.6 and the solution were transferred to a dialysis bag. The solution was dialyzed against deionized water (4 liters $\times$ 3) at 4°C over a period of 16 hours. The concentration of GC was determined by weighing the residue GC, obtained by slowly drying the GC solution at 60°C. When needed, GC solution was diluted with ultrapure pyrogen-free water or concentrated using a filtration cell. GC solution was autoclaved for 5 minutes at 121°C, and then cooled at room temperature and stored at 4°C until ready to use. The final GC product had a pH of 6.5; the addition of galactose made the chitosan soluble and slightly acidic.

The recovery of GC from the synthesis reaction was virtual 100%. NMR spectrum of GC revealed a small amount of galactol, a byproduct of the reaction which was removed by column chromatography during the final procedures of synthesis.

2. Animals and cell culture

Wistar Furth female rats were purchased (Harlan Sprague Dawley, Indianapolis, Indiana) at an age of six to ten weeks. The animals were used for the laser immunotherapy treatment of experimental animal tumors with GC as an immunoadjuvant as well as for the *in vivo* GC toxicity studies.

Three cell lines were used for the *in vitro* study of GC toxicity: EMT6 – a murine mammary tumor cell line, HeLa – a human epithelial carcinoma cell line, and VEC304 – a human normal cell line (vascular endothelial cell lines 304). Murine macrophage line RAW264.7 cells were used for the GC immunological stimulation study. The cells were cultured in RPMI 1640 (GIBCO) supplemented with 15% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 µg/ml) in 5% CO₂, 95% air at 37°C in a humidified incubator.

DMBA-4 mammary tumor cells were used for the laser immunotherapy treatment of animal tumors with GC as the immunoadjuvant. The preparation of the cells was the same as described above. The suspended cells (10⁵ cells in 0.1 ml solution per rat) were injected subcutaneously into one of the inguinal fat pads of each rat. When the tumor size reached 0.5 to 1.0 cm, the tumors were treated by laser immunotherapy. After treatment, the animals were housed individually. The animals were observed daily and the rat tumors were measured twice a week.

3. Determination of GC cytotoxicity *in vitro*

Cell toxicity assay was performed with a colorimetric tetrazolium salt-based assay, Cell Counting Kit-8 (CCK8). Tumor cells or normal cells (1×10^3 per well) were cultured in a 96-well microplate for 24 hours and then incubated with GC of different concentrations (0.005% to 0.625%) for 12 hours, rinsed with phosphate buffered saline (PBS), and then incubated for another 72 hours in complete medium. Using CCK8, the absorbance value at 450 nm, OD450, was read with a 96-well plate reader (INFINITE M200, Tecan, Switzerland) to determine the viability of the cells.

4. Determination of GC toxicity *in vivo*

A 1% aqueous solution of GC was substituted for drinking water for six Wistar Furth rats. The animals were observed daily. After continued feeding of GC for 75 days, the animals were sacrificed and an autopsy was performed for each animal.

GC solution of 1.15% was injected to the animals either subcutaneously or intraperitoneally. The animals were separated into three groups. Rats in one group were subcutaneously injected four times over a period of four weeks with 0.5-ml GC solution per injection. Rats in another group were injected intraperitoneally once with a 2.0-ml GC solution per injection. Two rats in the control group received no injection of GC solution. The injection protocol and GC doses are summarized in Table I. The animals were observed daily and after the final 0.5-ml GC injection, the animals were sacrificed and an autopsy was performed for each animal. The GC injection sites and vital organs were examined grossly and histologically.

5. Stimulation of TNF α secretion by GC

Macrophages used for the *in vitro* TNF α studies were 1×10^5 per well. Macrophages were incubated with GC of different concentrations in 24-well tissue culture plates. After 24 hours of incubation, the supernatants were collected and divided into different groups for TNF α ELISA detection using a commercially available kit (R&D Systems, Minneapolis, MN).

6. Laser immunotherapy treatment of mammary tumors in rats

Laser immunotherapy consists of three major components: a near-infrared laser, a light-absorbing dye, and an immunoadjuvant. In this study, the laser was an 805-nm diode laser, the dye was indocyanine green (ICG), and the immunoadjuvant was GC. The treatment protocol included an intratumoral injection of ICG (0.2 ml; 0.25%), GC (0.2 ml; 1.0%), or a mixture of ICG-GC (0.2 ml; 0.25% ICG and 1.0% GC). It constituted a dose of 10 mg/kg for GC and 2.5 mg/kg for ICG. The laser parameters were: 2 watts for 10 minutes. The rats were separated into eight groups and treated by different permutations of the three major components of laser immunotherapy. The experimental groups are shown in Table II.

RESULTS

GC was synthesized by incubating an aqueous suspension of chitosan chloride with a three-fold excess of galactose and subsequent stabilization by borohydride reduction of the mixture of Schiff bases and Amadori products (21). The procedure for the synthesis of GC is

briefly described in the Material and Methods. The diagram in Figure 1 shows the steps involved in the synthesis of GC.

After incubation in GC solutions of different concentrations, all tumor cells and normal cells remained viable, as shown in Figure 2A. Microscopic examination of control and GC-treated cells showed no morphological differences indicating no toxic effect at the cellular level (Figure 2B).

With the same diet given to the control animals and the experimental animals, no difference in body weight between the two groups was observed during the experiment. All the animals fed GC solution appeared healthy; no abnormalities or toxic effects were noted during daily observations. Autopsies were performed on all the rats. All the major organs, particularly the lungs, spleens, pancreases, and livers, were carefully observed grossly and histologically. There was no evidence of tissue abnormalities caused by the feeding of GC solutions.

The animals injected with GC solutions also behaved normally without any sign of toxic effects observed during the daily examinations. A complete autopsy was performed on each of the GC injected rats and nineteen major organs were grossly and histologically examined. The organs of rats injected with GC (either subcutaneously or intraperitoneally) did not show any evidence of drug toxicity. The only abnormalities observed were the multiple macroabscesses, surrounded by chronic fibrosis, attached to the capsular surface of the liver, as shown in Figure 3A, and chronic/active peritonitis surrounding the spleen, as shown in Figure 3B. These specific findings surrounding livers and spleens were due to acute immunological stimulation by GC injection, an expected inherent effect of an immunoadjuvant.

To determine the immunological effect of GC, we measured TNF α secretion by mouse macrophages, incubated with GC of different concentrations. As shown in Fig. 4, incubation with GC caused secretion of TNF α , and the level of TNF α secretion depended on the concentration of GC.

The survival data of the tumor-bearing rats after the treatment with different components of the laser immunotherapy protocol, individually or in combination, are shown in Table II. As shown by the data in Table II, survival tumor-bearing rats were all in the groups that received GC. The combination of all three components (ICG, GC, and laser irradiation) yielded the highest survival rate.

DISCUSSION

Chitosan is the partially N-deacetylated product of the natural polysaccharide chitin (1, 2). Due to its biocompatibility, biodegradability, and biological activity, chitosan has been widely used in biomedical applications (7–18). It has been reported that chitosan has an inhibitive effect on the growth of tumors (31). Chitosan also has adjuvant activity and can stimulate cytokine production in mice (32, 33) and augment the natural killer (NK) activity of mouse lymphocytes (34).

However, because of its intra- and inter-molecular hydrogen bonds (2), chitosan's rigid crystalline structure prevents it from being dissolved in water at pH near neutrality. Its poor water solubility limits the biomedical applications of chitosan.

Because of the presence of hydroxyl and primary amino groups in chitosan, it is easy to chemically modify chitosan to improve its solubility. Galactose was introduced to modify chitosan and the result was a new water-soluble compound, glycated chitosan (GC) (21). GC also has strong immunological effects in cancer treatment, as shown in the pre-clinical studies (22–26).

To further understand the physical and biological properties of GC, we conducted toxicity studies and immunological studies. To study GC's cytotoxicity, we used both normal cells and tumor cells (animal and human cell lines). Our results in Figure 2 show that even at high GC concentration (0.625%), all the cells remained viable. In our *in vivo* study, the feeding of GC did not induce any toxic effect in the animals. The injections of GC, either subcutaneously or intraperitoneally, did not result in any necrosis or apoptosis at injection sites or in different organs. Only minimal inflammatory effect, which produced moderate fibrosis and peritonitis surrounding livers and spleens, was observed, as shown in Figure 3. The histological studies showed no evidence of acute drug toxicity in any of the organs evaluated. Fibrosis and peritonitis surrounding the livers and spleens (Figures 3A and 3B) are consistent with an adjuvant effect, not drug toxicity.

When mouse macrophages were incubated with GC, TNF α secretion was increased, as shown in Figure 4. Such increase in secretion is clearly dose dependent. To demonstrate the immunological effect of GC *in vitro*, we choose TNF- α , a primary effector cytokine, produced by mouse macrophages upon stimulation of GC, as an example in this study. Other cytokines, some of which may be equal or more important in the induced immune responses, produced by macrophages of mice or rats upon GC stimulation, could further provide evidence for the immunological function of GC. We will carry such additional experiments in our future studies.

The effect of GC in cancer treatment is shown by the survival data of tumor-bearing rats after treatment by different components of laser immunotherapy (Table II). It is apparent that the use of GC increased the survival of the animals, due to its immunological stimulation. It is also clear that the combination of GC with photothermal therapy yielded the highest survival rate. It is interesting to notice that the treatment using laser+ICG, while the thermal effect was achieved, did not yield any survivors. The temperature elevation in the tumors treated by laser+ICG was significant (in many cases, exceeding 60°C). Furthermore, the tumor burden after laser+ICG treatment showed temporary reduction. These observations demonstrated the laser-ICG thermal tumor destruction. However, without the GC-enhanced immunological stimulation, the long-term anti-tumor effect was not achieved. This is a piece of indirect evidence that the immunological effects of GC play an important role in the treatment of the animal tumors. The laser-ICG-GC combination significantly increased the survival rates due to the synergistic effects of photothermal and immunological interactions.

The increased release of TNF α was attributed to the interaction between GC and the macrophages, although the exact signaling pathways have not been fully investigated. We plan to conduct such mechanistic studies. The selection of the low GC dose for the in vitro studies was due to the fact that we are working with only 1×10^5 cells, while the high GC dose for the in vivo studies was chosen based on several previous animal studies.

It should be pointed out that the starting molecular weight of the native chitosan was in the medium-range, about 500,000 molecular weight. The GC products may have a slight variation in the final molecular weight. However, our limited research with GC of different molecular weights has revealed no differences in either immune activity or toxicology.

We demonstrated in this study that GC is non-toxic in cell culture and in animal studies. The preliminary data suggest that GC could be safe to use in clinical applications. We also demonstrated the GC's ability in inducing immune response in cell culture and in animals. In summary, GC could be an effective immunoadjuvant for cancer treatment, particularly when combined with laser phototherapies.

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Figure 1.

Reaction of primary amino group of chitosan with the aldehyde group of galactose yields an Schiff base which is in equilibrium with its Amadori product. Subsequent treatment with sodium borohydride leads to the reduction of the double bonds and the formation of stable glycated chitosan (GC).

A **B** **Figure 2.**

Viability of cells treated with GC solution. (A) Cells (murine mammary tumor cell line, EMT-6; human breast tumor cell line, HeLa; human normal cell line, VEC-304) were incubated in GC solutions of different concentrations (0.005% to 0.625%) for 12 hours, then washed and incubated in complete medium for 72 hours before assessing cell viability. Cells not incubated with GC were used as control. Bars, SD (n = 4). (B) Optical images of EMT6 control tumors cells and cells treated with GC solution (0.625%). All cells (control and treated) appear normal.

A **B**

C **D** **Figure 3.**

Photomicrographs of different organs of a rat, treated with one intraperitoneal injection of 2 ml of 1.15% GC solution. Original magnification: 10 $\times$. (A) An essentially normal liver (lower right corner) and surrounding tissue. This photomicrograph reveals moderate congestion with minimal evidence of hepatocellular vacuolation, which is the same as the controls. Multiple macroabscesses surrounded by chronic fibrosis were mostly attached to the capsular surface of the liver (arrows). (B) An essentially normal spleen (lower right corner) and surrounding tissue. This photomicrograph reveals moderate extramedullary hematopoiesis similar to the controls, with no evidence of lymphoid follicular necrosis. The spleen is partially surrounded by chronic/active peritonitis, including abscess formation (arrow). (C) and (D) Photomicrographs of a normal liver (C) and spleen (D) of a control rat.

Figure 4.

TNFα secretion by macrophages incubated with GC detected by ELISA. Macrophages were incubated with GC solution of different concentrations (0.0025, 0.005, and 0.009%) for 24 h. After incubation, supernatants were collected for the determination of TNFα. Bars, SD (n = 4).

Table I

Animal experiments for GC injections.

Group	Number of Rats	GC Dose
Control	2	
GC Sub-Q Injection *	6	0.5 ml GC (1.0%) × 4
GC IP Injection **	6	2.0 ml GC (1.0%) × 1

* One injection per week for four weeks under the skin of one of front leg.

** One single intraperitoneal injection.

Table II

Survival of tumor-bearing rats after treatment with the components of the laser immunotherapy protocol.

Group	Number of Rats	Survival Rate *
ICG	12	0.0%
GC	12	8.3%
Laser only	12	0.0%
Laser + ICG	12	0.0%
Laser+ GC	12	8.3%
ICG + GC	12	16.7%
Laser + ICG + GC	31	29.0%
Control	35	0.0%

* Survival is defined as tumor-free for 120 days after tumor implantation.